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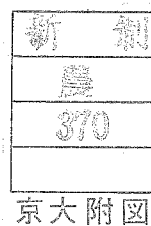
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STUDIES ON HEAT-INDUCED DNA INJURY AND  
ITS REPAIR IN *BACILLUS SUBTILIS*

YOSHIHIKO SAKO

1982

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## Chapter I

### INTRODUCTION

Bacterial spores are extremely resistant to various sterilization treatments, such as heating (1-3), ultraviolet irradiation (4),  $\gamma$ -ray irradiation (5), and some chemical agents (6-8), as compared with vegetative cells of the same strain and other microorganisms. Therefore, the bacterial spores must be sterilized very carefully in the processing and storage of foods.

Of various sterilization agents  $\gamma$ - and UV-irradiation are effective in some limited cases, but they cannot be used generally for many reasons. For example, the transmission of UV is very small, and it has not yet been established completely that  $\gamma$ -irradiated food is wholesome. Some chemical agents were widely used in preservation of foods before, but they are used in limited cases, because it has been pointed out that some chemical agents which had been used before were toxic or carcinogenic.

Then, the heat treatment which has been widely used in processing and storage of food from old times is now being watched with interest again. Thus the heating is thought to be a very important sterilization method in the food preservation. However, the molecular mechanisms of heat sterilization of bacteria, especially that of bacterial spores have scarcely been studied.

Many investigators have studied the survival rates and D values of spores of bacteria belonging to genera *Bacillus* and *Clostridium* heated at different temperatures, under different heating conditions since old time (3, 9-14). Recently, studies concerning the effects of mild heating on cellular components, such as membrane, enzyme proteins, rRNA and DNA, of vegetative cells of bacteria have been performed (15-30). In spores, however, the damages caused by heat treatment have scarcely been studied except in the case of enzyme proteins (31-33). As to DNA molecules in bacterial spores, there are few studies concerning the DNA injury and its repair mechanisms, because of the difficulty of mild extraction of DNA from spores. It has been known that DNA molecule is the most important target in  $\gamma$ - and UV-irradiation (34-36). But the action site of heat in bacterial cells has not yet been made clear.

In purified DNA, Tamm et al. (37) found for the first time that acid treatment of DNA protonated the N-glycosidic bonds of purines resulting in hydrolysis of purines from the pentose sugar without breaking the phosphodiester backbone directly (Fig. 1). Furthermore, Greer and Zamenhof (38) found that purine bases are also slowly released from DNA during incubation at neutral pH and high temperature. Several investigators (39, 40) reported that mutations were induced in spores of *B. subtilis* by heating at high temperatures. Alteration of requirements for germination in heat-injured spores of *B. stearothermophilus* was also reported (41). Therefore, most of the early

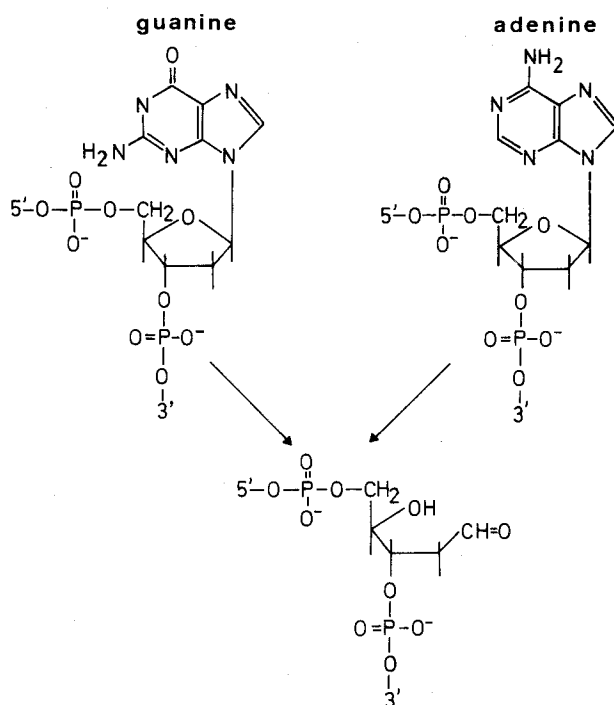


Fig. 1. Scheme for depurination by heating.

studies attributed heat mutagenesis to depurination (38,39,42,43). On the other hand, Drake and co-workers (44-46) showed that GC - AT transition and GC - CG transversion were induced by heat and the depurination was probably little or not mutagenic in bacteriophage T4. In this case, however, the phage was heated at relatively low temperature and for long time. Recently, it has been shown that DNA polymerase can copy past apurinic sites on natural, biologically active DNA and further, that this leads to increased mutagenesis (47, 48). At high temperatures as used for sterilization of foods or near the  $T_m$  of DNA, the heating was reported to produce apurinic sites in *in vitro* DNA (38, 49). Therefore, it is expected that heat



treatment at high temperatures induces damages of DNA molecules in spores, such as depurination or depyrimidination, and the damaged DNA molecules in spores are repaired during germination of spores under appropriate conditions.

Recently, it has been found that many alkylating and carcinogenic agents modify the bases of DNA and the modified bases are depurinated or depyrimidinated by specific DNA glycosylases (50-52). Accordingly, as a repairing enzyme, endonuclease specific to apurinic sites in DNA (APendodeoxyribonuclease) was paid attention to and extensively investigated. APendodeoxyribonucleases have been isolated from *Escherichia coli* (53-55), *Hemophilus influenzae* (56), *Micrococcus luteus* (57, 58), *Bacillus subtilis* (59), *Bacillus stearothermophilus* (60), higher plant (61), and animals (62-64). These endonucleases have been studied in relation to the DNA injuries induced by alkylating agents, X-ray irradiation, ultraviolet irradiation, or carcinogenic substances, and their repair systems.

In order to make clear the repair system in heat-injured DNA in spores, which perhaps involves depurination reaction, it is necessary to purify and characterize the deoxyribonuclease specific to heat-injured DNA.

The present study was attempted to make clear the following questions ; [1] whether or not mutations are caused by heat treatment in *B. subtilis* spores, [2] whether or not DNA injury of *B. subtilis* can be detected by sedimentation analysis and DNA damage is due to depuri-

nation, [3] whether or not heat induced DNA injury of spores is different from that of vegetative cells, [4] whether or not injured DNA is repaired during incubation after heating, [5] whether or not the repair enzyme specific to the heat-damaged DNA is present in *B. subtilis*.

## Chapter II

### APPEARANCE OF MUTANTS BY HEAT TREATMENT OF *BACILLUS SUBTILIS* SPORES

#### 1. INTRODUCTION

The bacterial spores are completely inactivated by heat treatment at very high temperature, but they may only be partially inactivated when they were treated with heat under not so drastic conditions. Recent reports have indicated that the heat treatments of *Clostridium* and *Bacillus* spores may result in (i) dead spores, (ii) injured survivors, (iii) uninjured survivors (63-72). Heat treatment may damage either enzyme systems in the spores or specific sites on the chromosome which are essential for germination and outgrowth. Under suitable conditions, the injured spores may be recovered and behave as uninjured spores.

In this chapter, the effect of addition of amino acids to the germination media on the recovery of the heat-injured spores of *Bacillus subtilis* was investigated.

#### 2. MATERIALS AND METHODS

##### Organism

*Bacillus subtilis* ATCC 6051 was used throughout this work.

### Preparation of spores

*B. subtilis* was grown at 37°C in the nutrient broth containing 0.5 % peptone (Polypeptone, Daigo) and 0.5 % beef extracts (Kyokuto), supplemented with potato extract. Spores were harvested after 48 h incubation and then purified by use of the two-phase Y system which was reported by Sacks and Alderton (73). The purified spores were thoroughly washed with distilled water and lyophilized, and then stored in a desicator at -20°C.

### Heat treatment and counting of the survivors

Spores suspended in distilled water (0.15 mg of dry spores/ml) were heated at 80, 90, or 100°C in water baths. After being heated for desired periods the spore suspensions were immediately cooled in ice-water.

Schaeffer's agar medium (74) and Demain's minimal agar medium (75) were used for counting the spores which survived the heat treatment. Schaeffer's agar medium was prepared to contain 8 g nutrient broth, 1 g KCl, 0.25 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.25 mg  $\text{MnCl}_2$ ,  $10^{-3}$  M  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ,  $10^{-6}$  M  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g agar in 1000 ml. Demain's minimal agar medium was prepared to contain 1 g glucose, 890 mg L-alanine, 1470 mg L-glutamic acid, 1320 mg L-asparagine, 3 g  $\text{K}_2\text{HPO}_4$ , 1 g  $\text{KH}_2\text{PO}_4$ , 500 mg  $\text{NH}_4\text{Cl}$ , 100 mg  $\text{NH}_4\text{NO}_3$ , 100 mg  $\text{Na}_2\text{SO}_4$ , 10 mg  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 mg  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 1 mg  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 mg  $\text{CaCl}_2$ , 15 g agar in 1000 ml.

The spores not being heated germinated and grew well in the Demain's minimal agar medium. Amino acid to be tested was supple-

mented to the Demain's minimal agar medium in the concentration of 10 mM. The number of colonies grown on the agar plates was counted after the incubation at 37°C for 48 h.

### 3. RESULTS

#### Injury of spores by heating and their recovery

The heat survival curves of *B. subtilis* spores were made by use of Schaeffer's agar medium and Demain's minimal agar medium after being heated at 80, 90, and 100°C for different periods (Fig. 2). Logarithms of the number of survivors were plotted against the heating time. Vial counts of unheated controls were the same on Schaeffer's or Demain's medium. The lower the heating temperature, the more the spores were viable in both agar media. When the spores were heated at 80°C, the survival fraction after 20 min was almost 100 % on Schaeffer's medium but was only approximately 8 % on Demain's medium. In the case of heating at 90°C for 20 min, about  $10^{-2}$  of the total spores grew on Schaeffer's medium whereas  $10^{-6}$  grew on Demain's medium. At 100°C numbers of survivors on Schaeffer's medium and Demain's medium were similar. These results indicated that some of the spores injured at temperature below 90°C were able to recover when suitable cultural conditions prevailed ; however, spores heated at 100°C showed no recovery at all.

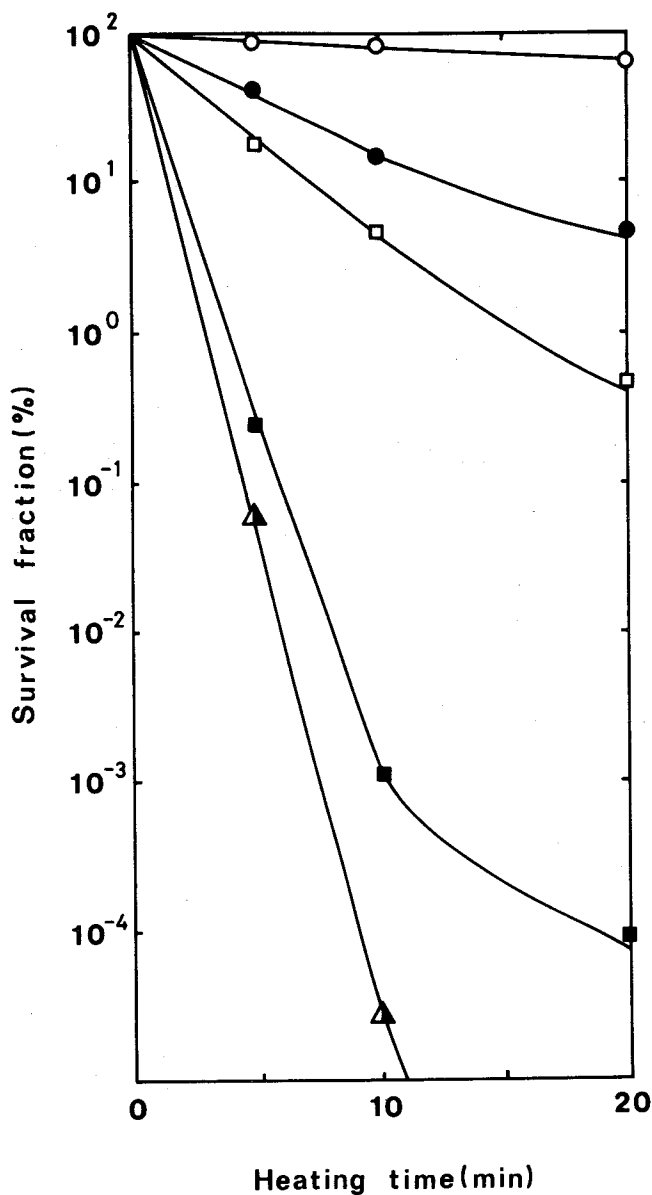


Fig. 2. Heat-survival curves of *B. subtilis* spores on Schaeffer's agar and Demain's minimal agar medium.

Symbols : ○—○, heated at 80°C and plated on Schaeffer's agar ; ●—●, heated at 80°C and plated on Demain's agar ; □—□, heated at 90°C and plated on Schaeffer's agar : ■—■, heated at 90°C and plated on Demain's agar : △—△, heated at 100°C and plated on Schaeffer's agar ; ▲—▲, heated at 100°C and plated on Demain's agar.

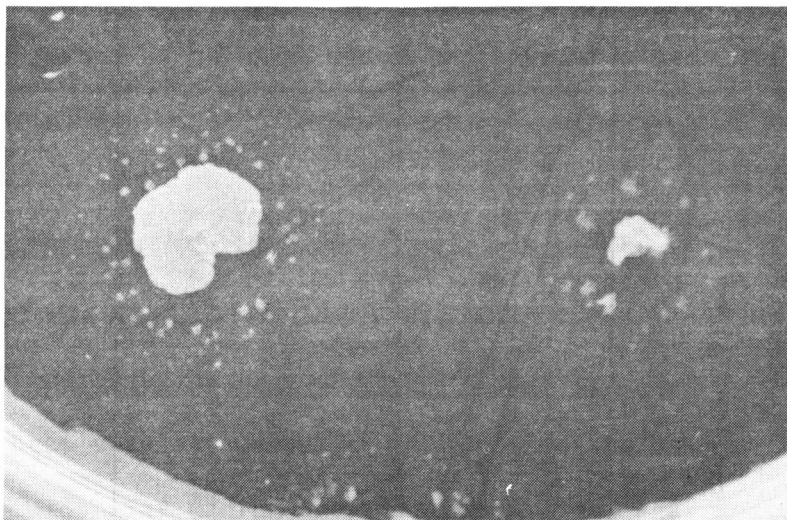


Fig. 3. Colonies formed from heated (90°C, 10 min) spores of *B. subtilis* on <sup>a</sup>Demain's minimal agar plate (incubated at 37°C for 4 days).

In the case of spores heated at 90°C for 10 min, small satellite colonies often appeared on Demain's agar medium around a big colony which probably originated from an uninjured spore (Fig. 3). This fact suggests that the big colony excreted some substances which repaired the heat-injured spores.

#### Effect of amino acid supplementation on recovery

The principal difference in composition between Schaeffer's medium and Demain's medium is that the former contains most amino acids but the latter contains only alanine, glutamic acid, and asparagine. To analyze the effect of Schaeffer's medium on recovery of heat-injured spores, the survivors of the heat-injured spores were counted by use of the Demain's minimal agar medium supplemented by various

groups of amino acids (Table 1). As shown in Table 1, the supplement of casamino acids or some groups of amino acids to Demain's minimal agar medium resulted in the significant restoration of the heat-injured spores which were unable to germinate and to grow without the supplement. Especially, some amino acids contained in groups I and VI were effective. The effect of individual amino acids on recovery was further examined to determine which amino acid was responsible for recovery of the heat-injured spores. The results obtained (Table 2) indicated that only glycine and threonine were effective in the recovery of the heat-injured spores. None of the

Table 1. Effect of addition of amino acid mixtures to counting media on the number of *B. subtilis* spores surviving after being heated at 90°C for 10 min.

Medium	Non. of survivors
Schaeffer's medium	$1.4 \times 10^6$
Demain's minimal medium (DMM)	$1.3 \times 10^3$
DMM + Casamino acids	$1.4 \times 10^6$
DMM + group I	$8.6 \times 10^5$
DMM + group II	$1.5 \times 10^3$
DMM + group III	$1.0 \times 10^2$
DMM + group IV	$1.1 \times 10^3$
DMM + group V	$2.3 \times 10^3$
DMM + group VI	$1.3 \times 10^6$

Group I : glycine, L-phenylalanine, L-methionine.

Group II : L-valine, L-leucine, L-tryptophan.

Group III : L-lysine, L-serine, L-cysteine.

Group IV : L-isoleucine, L-proline, L-tyrosine.

Group V : L-histidine, L-arginine, L-glutamic acid.

Group VI : L-aspartic acid, L-threonine.

Each amino acid was added to the Demain's minimal agar medium so as to be 10 mM at the final concentration.



other amino acids tested was effective in this respect. However, homoserine, which is an intermediate substance in the threonine biosynthesis pathway, had the same effect as glycine or threonine (data were not shown). These results suggest that some sites in amino acid metabolism, probably those involved in the metabolism between glycine and serine and/or that between aspartic acid and homoserine, were injured by heating at 80 or 90°C.

#### Appearance of glycine auxotrophs after heat treatment

In Fig. 4, logarithms of the numbers of survivors and spores which required glycine for germination and outgrowth were plotted against the heating temperature after heating for 10 min. The sur-

Table 2. Effect of addition of individual amino acids to counting media on the number of *B. subtilis* spores surviving after being heated at 90°C for 10 min.

Medium	No. of survivors
Schaeffer's medium	$1.4 \times 10^6$
Demain's minimal medium (DMM)	$1.3 \times 10^3$
DMM + glycine	$1.3 \times 10^6$
DMM + L-phenylalanine	$1.4 \times 10^3$
DMM + L-methionine	$1.2 \times 10^3$
DMM + L-aspartic acid	$1.2 \times 10^3$
DMM + L-threonine	$1.5 \times 10^6$

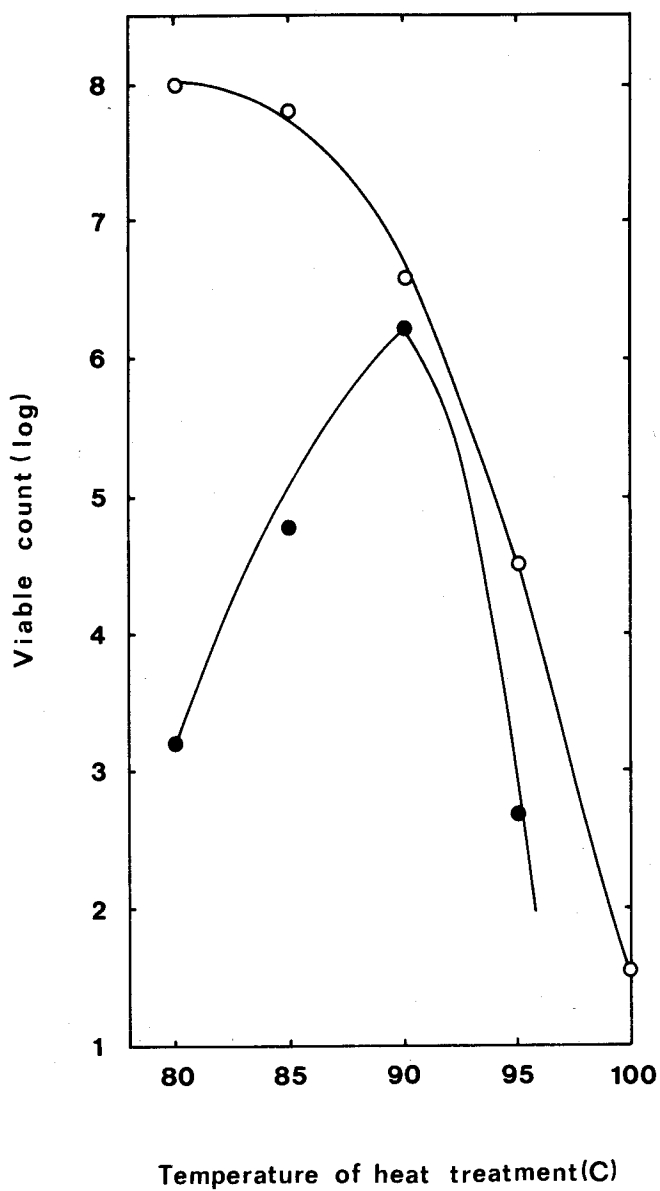


Fig. 4. Development of glycine-requiring mutants of *B. subtilis* by heat treatment at various temperatures.

Symbols : ○—○, survivors ; ●—●, glycine-requiring mutants.

The heat treatment was for 10 min.

vival fractions decreased exponentially with the increase in temperature. At 90°C a large percentage of the spores became glycine requiring, and the morphologies of colonies of the glycine requiring cells were different from those of the other cells. Colonies of the heated spores, most of which were glycine requiring, grown on Demain's minimal agar medium supplemented with glycine were very small and amorphous.

The glycine auxotrophy of the cells was maintained after more than five transfers to new agar plates, which indicates that the amino acids requirements of spores induced by the heat treatment were genetically inherited.

#### 4. DISCUSSION

The spores of *Bacillus subtilis* which were injured by heat treatment at 90°C for 10 min were not able to germinate in Demain's minimal agar medium. However, the injured spores underwent recovery and germinated when the medium was supplemented with glycine, threonine or homoserine. On the other hand, when the spores were heated at 100°C, the survival curve obtained with Schaeffer's agar medium was almost the same as that obtained with Demain's minimal agar medium, namely the spores heated at 100°C could not restore the viability at all.

Edwards et al. (76) reported that dipicolinate and  $\text{CaCl}_2$  were effective in the recovery of heat-injured spores of *B. subtilis*.

Campbell et al. (41) found that glutamic acid and lysine were effective in enhancing the recovery of heated spores of *B. stearothermophilus*. Yokoya and York (77), and Cook and Brown (78) also reported that a high D value was obtained with *B. coagulans* and *B. stearothermophilus*, respectively, when 1 % starch was added to the counting medium.

Zamenhof (39) also found that spores which survived heat treatment formed small colonies on plates. Chiasson and Zamenhof (40), and Northrop and Slepecky(79) reported that, when spores of *B. subtilis* were heated (80°C to 100°C) and then plated, numerous mutants were obtained and these mutants exhibited abnormal sporulation. They proposed that these mutations were due to depurination. Our results were consistent with their hypothesis, since we observed that the spores were inactivated exponentially with an increase in dosage of heat and that most of the spores which survived the heat treatment at 90°C became auxotrophic.

Since the amino acid requirements of spores induced by the heat treatment are genetically inherited, it is suggested that the heat treatment is accompanied by modification of a specific site on the spore chromosome.

## 5. SUMMARY

The spores of *B. subtilis* which had been injured by heat treatment at 90°C for 10 min could not form colonies on Demain's minimal agar medium. The injured spores, however, recovered and grew when the medium was supplemented with glycine, threonine, or homoserine. The amino acids requirements of spores induced by the heat treatment were genetically inherited. It is suggested that the heat treatment is accompanied by the modification of a specified site on chromosome in the spores.

## Chapter III

### DNA INJURY BY HEAT TREATMENT IN *BACILLUS SUBTILIS*

#### Section 1. DEATH AND DNA INJURY OF SPORES BY HEAT TREATMENT

##### 1. INTRODUCTION

The heat resistance of bacterial spores depends on the inherent genetic factors (80-82), growth temperature (83), buffer solution in which spores are suspended (84-86), concentration of spores (87), heating condition (88-89), and plating medium (69,90) etc. When the spores are harvested and plated after heating under the same conditions, the heat resistance of spores is mainly determined by the pH values of spore suspension exposed to heat.

Xezones and Hutchings (91) studied the heat resistance of *Clostridium botulinum* spores and found that the decimal-reduction time (D value) of the spores increased with an increase in pH and the influence of pH was pronounced at lower heating temperature. Löwik and Anema (92) found that when the pH was decreased from 6.0 to 4.8 the D value was decreased by 40 %, irrespective of the heating temperature, and z value was independent of pH. On the other hand, Cameron et al. (93) recently reported that increasing acidity was, in general, accompanied by decreasing heat resistance of *Clostridium sporogenes*

spores, although pH effect was more pronounced at the higher processing temperature. The effects of low pH on the thermal resistance of spores were studied by several investigators in relation to the sterilization of foods (94-96).

In recent years, the biochemical nature of the lesions caused by heating of bacteria under sublethal and lethal temperature conditions has attracted attention of investigators. Although the heat-induced injuries in bacterial spores have been reported (36, 97-100), most of those studies have dealt with membrane, protein, and RNA. And concerning the DNA injury in heated spores few studies have been made.

Then, in this chapter, the author studied the effect of acid-heating on the survival of *B. subtilis* spores and the heat-injury of DNA in the spores.

## 2. MATERIALS AND METHODS

### Organism

*B. subtilis* 168Tt (thy<sup>-</sup>, trp<sup>-</sup>) was used throughout this work.

### Preparation of nonlabeled and <sup>3</sup>H-labeled spores

*B. subtilis* was grown at 37°C in Schaeffer's medium (74) and spores were harvested after 48 h incubation. The spores were purified by treatment with 500 µg of lysozyme per ml at 37°C for 40 min and 1 % sodium dodecyl sulfate at 37°C for 20 min. The spores were washed several times with distilled water and stored at 0°C. Spores

labeled with  $^3\text{H}$  were harvested after 48 h incubation in Schaeffer's medium supplemented with 250  $\mu\text{Ci}$  of [methyl- $^3\text{H}$ ] thymidine (58 Ci/mmol : New England Nuclear) per 100 ml and were purified by the same method used in nonlabeled spores.

#### Heat treatment and counting of the survivals

Spores suspended in distilled water were centrifuged and the pellet was suspended again in M/20 sodium citrate-HCl buffer (pH 3, 5, 7). Spore suspension in buffer was heated at 90, 80, 70, or 60°C in water bath and was immediately cooled in ice bath. The nutrient agar medium containing 0.5 % peptone, 0.5 % beef extracts and 1.5 % agar was used for the enumeration of the spores which survived the heat treatment. Colonies were counted after incubation at 37°C for 24 h.

#### Extraction of DNA from unheated and heated spores

Unheated or heated  $^3\text{H}$  labeled spores were treated with 2 % sodium dodecyl sulfate in 0.5 N NaOH at 37°C for 1 h and washed two times with distilled water. Then spores were treated with 1 mg/ml lysozyme in 0.01 M Tris-HCl (pH 8.0), 0.01 M EDTA-4Na, 0.2 % Brij-58 and 10 % sucrose at 37°C for 30 min and centrifuged. The pellet was resuspended and solubilized slowly in 2 % sodium dodecyl sulfate in 0.1 N NaOH at 37°C for 30 min and then DNA was extracted.

#### Sedimentation analysis of $^3\text{H}$ labeled DNA in alkaline sucrose density gradient

Extracted DNA was layered on linear 5 - 20 % alkaline sucrose



density gradient (pH 12.0) and centrifuged at 40,000 rpm for 2 h at 20°C in a Beckman SW. 50.1 rotor. The contents were collected from the top in fractions of 0.15 ml on the disks of filter paper with ISCO density gradient fractionator. The disks were dried and radioactivity was counted in toluene system.

### 3. RESULTS

#### Heat resistance of spores to acid-heat treatment

The thermal death curves resulting from acid-heat treatment of *B. subtilis* spores at 90, 80, 70, 60°C were given in Fig. 5.

Logarithmus of the number of survivors after heat treatment in the citrate buffer adjusted to pH 7.0, 5.0, and 3.0 were plotted against heating time.

When the spores were heated at 90°C, the survival fractions after 5 min heating at pH 7.0, 5.0, and 3.0 were about 60, 2, and 0.004 % respectively. In the case of heating at 80°C, the survivals after 20 min heating at pH 7.0, 5.0, and 3.0 were about 60, 8.3, and 0.008 % respectively. At 70°C for 20 min, the survivals after heating at pH 7.0, 5.0, and 3.0 were about 90, 60, and 4.6 % respectively. At 60°C for 20 min, the survivals after heating at pH 7.0, 5.0, and 3.0 were about 95, 72, and 50 % respectively.

These results indicated that at the same temperature spores were more resistant at neutral pH than at lower pH. Consequently, at the

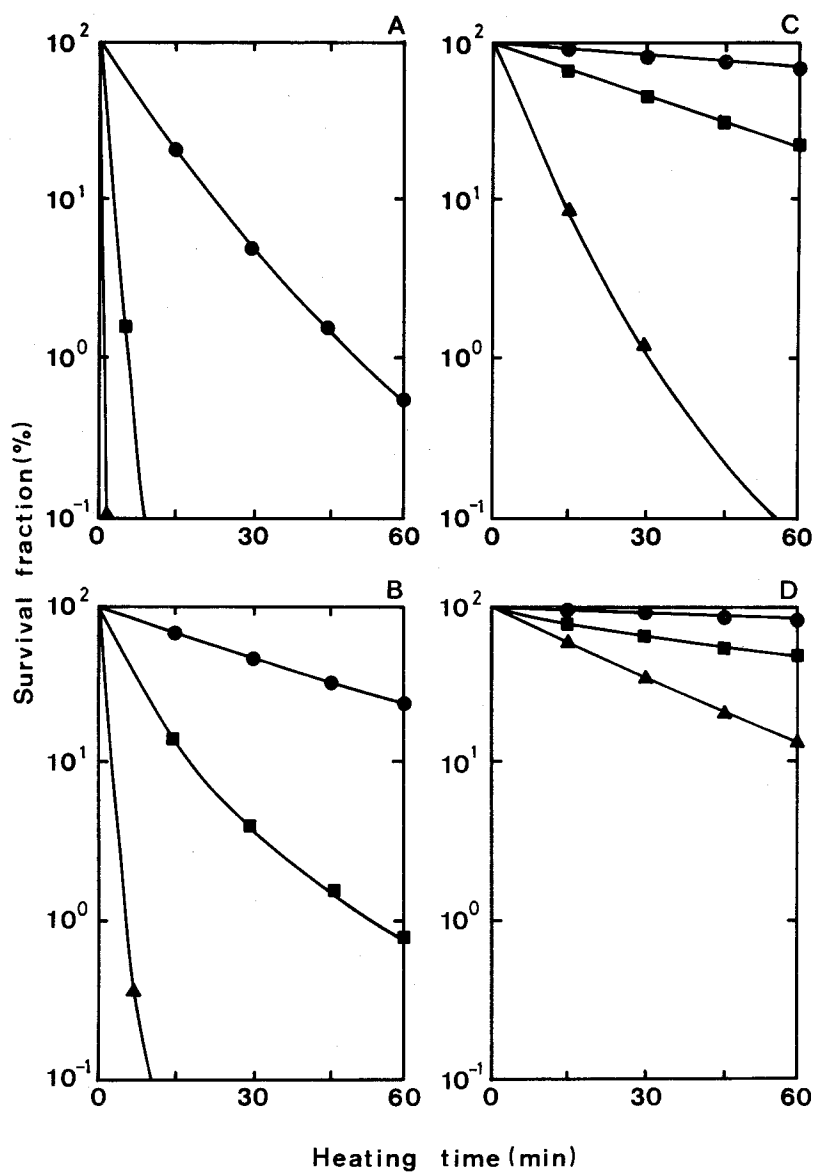


Fig. 5. Thermal death curves of *B. subtilis* spores.

A, 90°C ; B, 80°C ; C, 70°C ; D, 60°C.

Symbols : ●—●, heated at pH 7.0 ; ■—■, heated at pH 5.0,  
▲—▲, heated at pH 3.0.

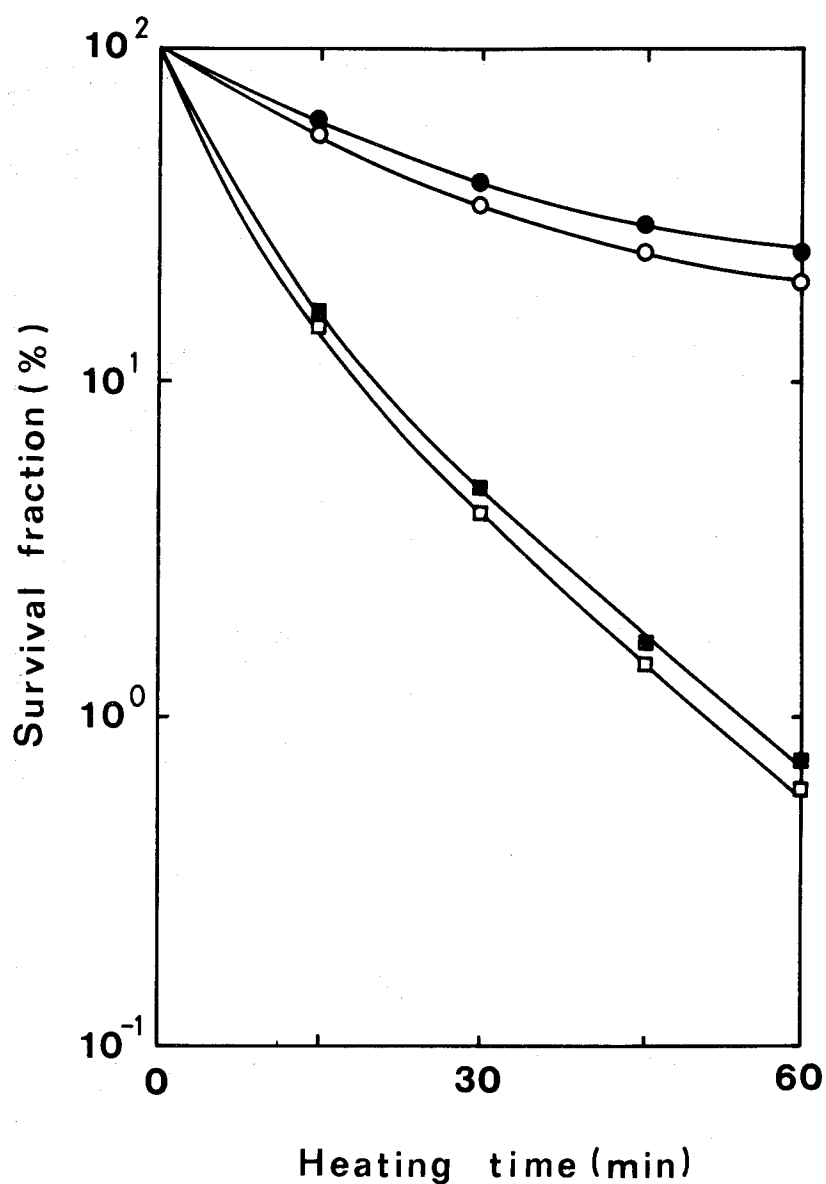


Fig. 6. Effect of incubation time at pH 5.0 on thermal destruction of *B. subtilis* spores heated at 80°C.

Symbols : ●—●, heated at pH 7.0 ; ■—■, heated at pH 5.0 ; ○—○, dialyzed against sodium citrate-HCl buffer (pH 5.0) for 24 h at 5°C and washed, and then resuspended in sodium citrate-HCl buffer (pH 7.0) and heated ; □—□, dialyzed against sodium citrate-HCl buffer (pH 5.0) for 24 h at 5°C and heated.

same pH the higher temperature, the more the spores were inactivated. Therefore, the lower pH and the higher temperature, the more the spores were inactivated.

To examine the effect of incubation time at pH 5.0 on survival of spores, the spores suspension ( $1 \times 10^8$  spores/ml) was dialyzed against sodium citrate-HCl buffer (pH 5.0) for 24 h at 5°C and washed, and then resuspended in sodium citrate-HCl buffer (pH 7.0) just before the heating at 80°C. Fig. 6 shows that the thermal death curve of spores incubated at pH 5.0 for 24 h was almost the same as those of spores suspended in the buffer just before the heating.

Similarly in the case of pH 3.0, the same result was obtained. These results indicates that the water surrounding the spores may penetrate into the spore inside at just time when the spores are heated.

#### Injury of DNA molecules induced by acid-heating in spores

To examine the DNA injury in spores by acid-heating, DNA extracted from heated spores was analyzed in respect to the molecular weight, by using alkaline sucrose gradient sedimentation. Sedimentation patterns of DNA in spores heated at 90, 80, 70, and 60°C were given in Figs. 7, 8, 9, and 10, respectively.

Fig. 7 A shows that single strand scissions of DNA molecules in spores heated at 90°C at pH 7.0 did not occur even after heating for 30 min, but at low pH single strand breaks of DNA increased according to the increasing acidity. Fig. 7 B shows that after 5, 10, and 15

min of incubation at 90°C at pH 5.0, the sedimentation distance of DNA decreased with the increase of heating time.

Fig. 8 A shows that single strand scissions in DNA molecules in spores heated at 80°C at pH 7.0 did not occur even after heating for 30 min, but at low pH DNA breaks increased according to the increasing acidity. Fig. 8 B shows that after 5, 10, and 15 min of incubation at 80°C at pH 5.0, the sedimentation distance of DNA decreased with the increase of heating time. The number of DNA breaks induced by heating at 90°C is more than at 80°C. Fig. 9 A similarly shows that single strand scissions of DNA in spores heated at 70°C at pH 7.0 did not occur even after heating for 30 min, but at low pH DNA breaks increased according to the increasing acidity. Fig. 9 B shows that after 15, 30, and 60 min of incubation at 70°C at pH 5.0, the sedimentation distance of DNA decreased with the increase of heating time. Fig. 10 A shows that single strand scissions of DNA in spores heated at 60°C at pH 7.0 and pH 5.0 did not and few occur, respectively, but at pH 3.0 DNA scissions occurred. Fig. 10 B shows that after 15, 30, and 60 min of incubation at 60°C at pH 5.0, the sedimentation distance of DNA similarly decreased with the increase of heating time, however, the number of DNA breaks induced at 60°C was fewer than at higher temperature.

These results show that after heating at 90, 80, 70, or 60°C at neutral pH, single strand scissions in DNA did not occur even after heating for 30 min. When at the lower pH and the higher temperature

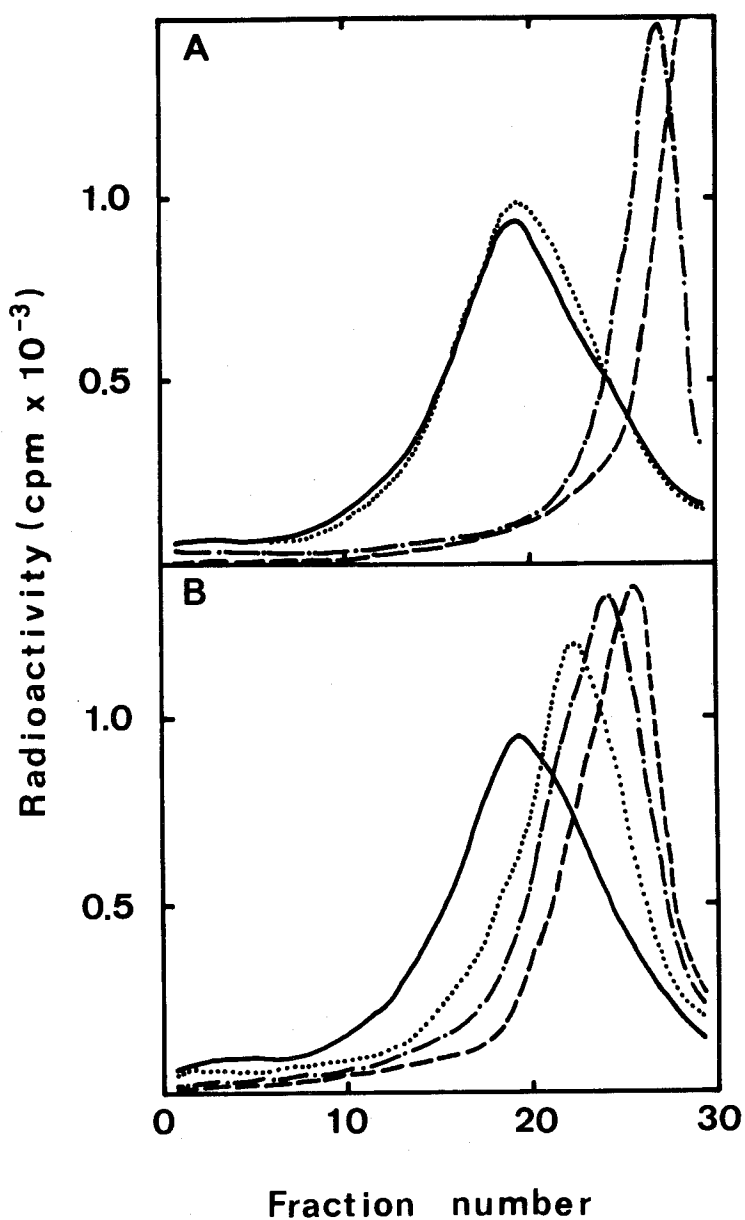


Fig. 7. Sedimentation patterns in alkaline sucrose density gradients of DNA from *B. subtilis* spores heated at 90°C for 30 min (A) and at pH 5.0 (B). The direction of sedimentation is from right to left. (A) : —, non-heated ; ·····, heated at pH 7.0 ; — —, heated at pH 5.0 ; — — —, heated at pH 3.0. (B) : —, non-heated ; ·····, heated for 5 min ; — —, heated for 10 min ; — — —, heated for 15 min.

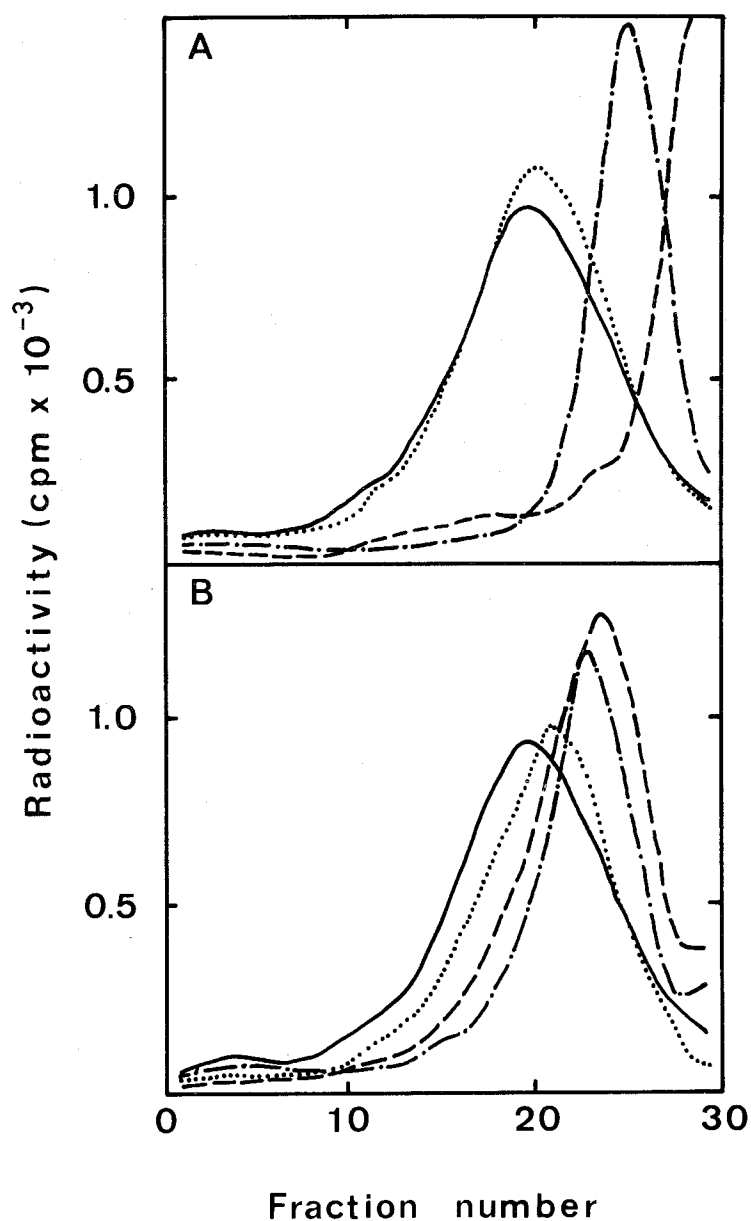


Fig. 8. Sedimentation patterns in alkaline sucrose density gradients of DNA from *B. subtilis* spores heated at 80°C for 30 min (A) and at pH 5.0 (B). The direction of sedimentation is from right to left. (A) : —, non-heated ; ·····, heated at pH 7.0 ; —·—, heated at pH 5.0 ; ---, heated at pH 3.0. (B) : —, non-heated ; ·····, heated for 5 min ; —·—, heated for 10 min ; ---, heated for 15 min.

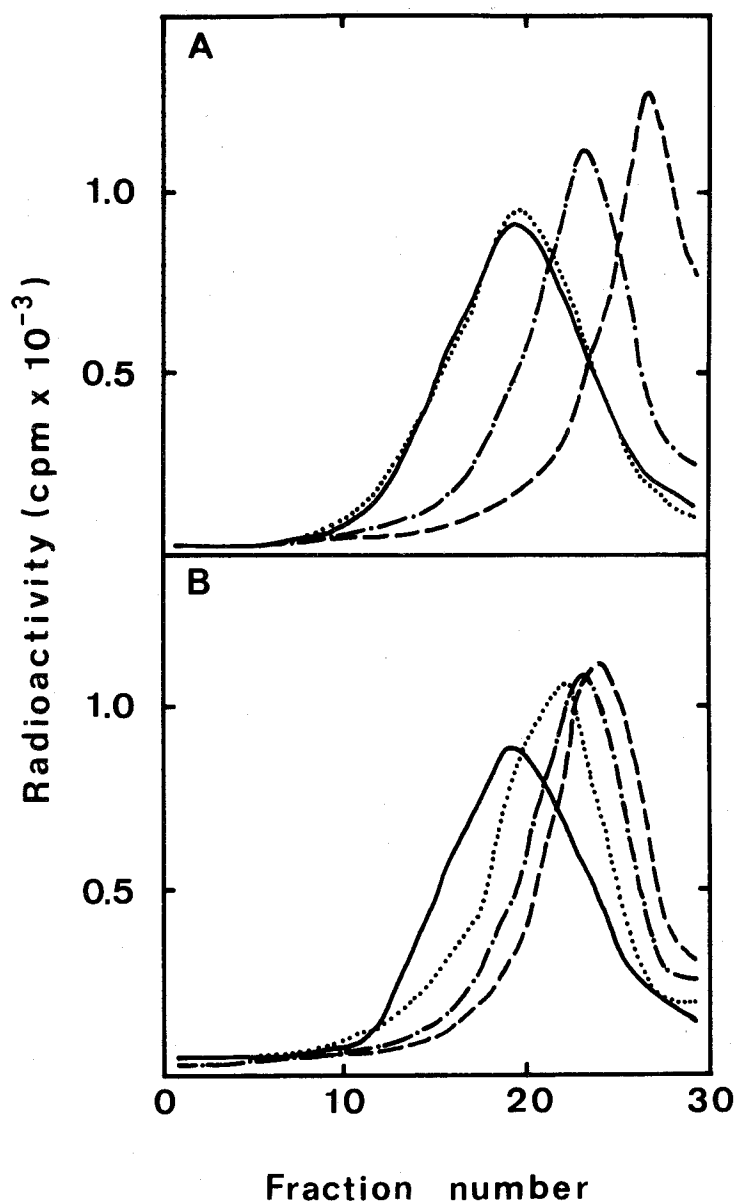


Fig. 9. Sedimentation patterns in alkaline sucrose density gradients of DNA from *B. subtilis* spores heated at 70°C for 30 min (A) and at pH 5.0 (B). The direction of sedimentation is from right to left. (A) : —, non-heated ; ·····, heated at pH 7.0 ; —·—, heated at pH 5.0 ; ---, heated at pH 3.0. (B) : —, non-heated ; ·····, heated for 15 min ; —·—, heated for 30 min ; ---, heated for 60 min.



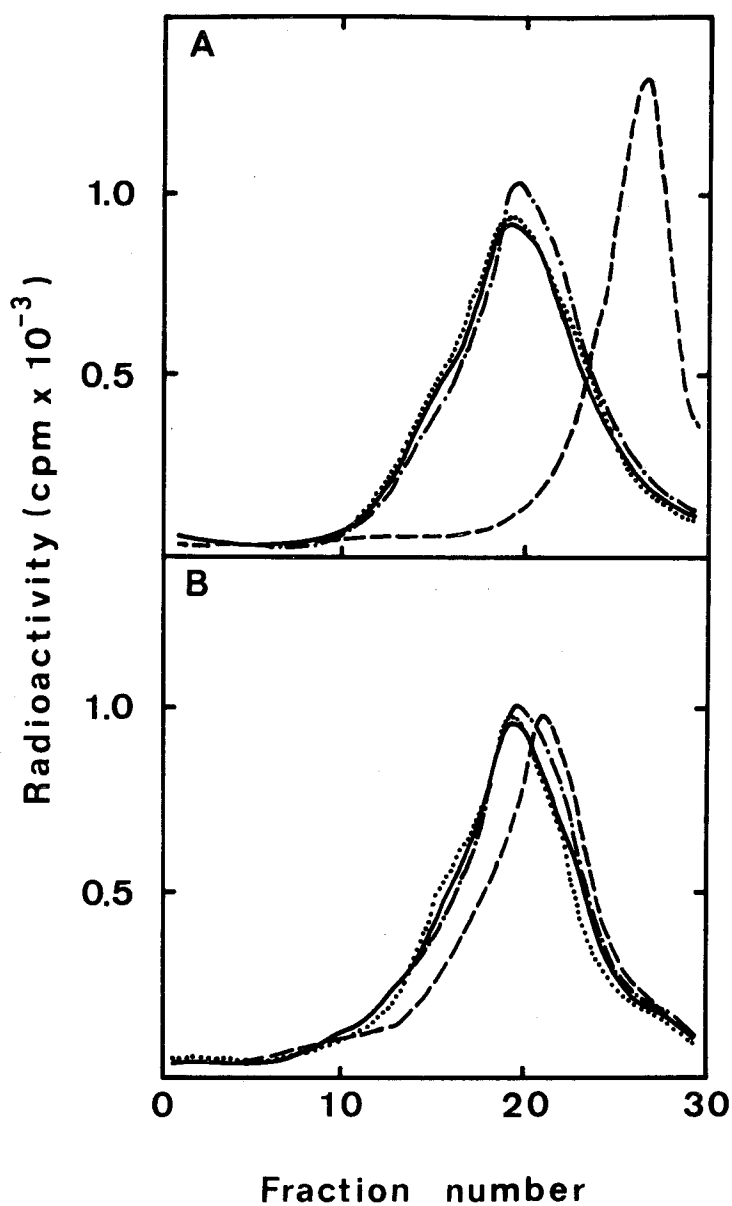


Fig. 10. Sedimentation patterns in alkaline sucrose density gradients of DNA from *B. subtilis* spores heated at 60°C for 30 min (A) and at pH 5.0 (B). The direction of sedimentation is from right to left. (A) : —, non-heated ; ·····, heated at pH 7.0 ; —·—, heated at pH 5.0 ; ---, heated at pH 3.0. (B) : —, non-heated ; ·····, heated for 15 min ; —·—, heated for 30 min ; ---, heated for 60 min.

the spores were heated, the more the single strand scissions of DNA in spores occurred.

It is reported that the main injury of *in vitro* DNA caused by heat treatment is depurination (38) and depurination occurred more frequently at acidic pH than at neutral pH (47). It is generally said that the apurinic sites in DNA are labile to the alkaline treatment resulting chain breaks. Accordingly, it is thought that the apurinic sites in DNA molecules induced by acid-heat treatment of spores, but not at neutral pH, were attacked by the alkaline treatment for the sedimentation analysis and the chain breaks were introduced.

#### 4. DISCUSSION

The effect of low pH on the thermal resistance of *Clostridium botulinum* spores was well reported in relation to the putrefaction of food products and the toxin production. It is a generally accepted fact that a pH of 4.6 or less inhibit the growth and toxin production of *C. botulinum* spores in food (101). Xezones and Hutchings (91) found greater pH effect at the lower rather than at the higher temperature.

On the other hand, Cameron et al. (93) showed that the heat resistance of spores decreased with decreasing pH, but the pH effect was pronounced at the higher processing temperature than the lower

temperature.

The heating causes the damages either enzymes or DNA in spores which are essential for germination and outgrowth. It has been also reported that heat treatment at high temperatures often induced mutation in spores of *B. subtilis* (39, 40). In the previous chapter, it was found that heat treatment of *B. subtilis* spores induced the mutants which required amino acids for germination and/or growth.

Therefore, in this chapter, the effects of heat treatment, especially acid-heating, on survivals and DNA injury in spores were studied. As the results, the heat resistance of spores decreased with decreasing pH. Although after heating of spores at 60 - 90°C at neutral pH single strand scissions of DNA molecules did not occur even after heating for 30 min when at the lower pH and the higher temperature the spores were heated, the more the single strand scissions of DNA in spores occurred.

Greer and Zamenhof (38) reported that depurination occurred by heat treatment of DNA at high temperature. Furthermore, Lindahl and Nyberg (47) found that apurinic sites were induced more frequently at acidic than neutral pH. Accordingly, it is strongly suggested that apurinic sites in DNA molecules induced by acid-heat treatment of spores were attacked by the alkaline treatment and phosphodiester bonds at the apurinic sites were broken.

These results indicated that DNA molecules in spores was damaged, perhaps depurination, by heat treatment at acidic pH.

## 5. SUMMARY

The spores of *B. subtilis* suspended in buffer solution of low pH were more sensitive to heat than those in neutral pH. The correlation of the heating temperature (60, 70, 80, 90°C) and pH value in the range from 3 to 7 on the thermal destruction of spores of *B. subtilis* was examined. When treated at the lower pH and the higher temperature, the more the spores were inactivated.

To examine the effect of acid-heating on DNA in spores, after heating DNA was extracted from spores and analyzed by alkaline sucrose gradient sedimentation. The results indicated that when the spores were heated at the lower pH and the higher temperature, the more the single strand scissions of DNA in spores occurred. It is thought that depurination occurred first by the acid-heat treatment and then the DNA was broken at apurinic sites by the subsequent alkaline treatment.

## Chapter III

### Section 2. DEATH AND DNA INJURY OF VEGETATIVE CELLS BY HEAT TREATMENT

#### 1. INTRODUCTION

As shown in the previous chapter, it was found that the heat treatment caused DNA injury in *B. subtilis* spores. Especially, the acid-heat treatment caused the single strand scissions of DNA molecules, perhaps through the depurination, in spores.

It is expected that such scissions of single strand DNA molecules caused by heating can also be found in vegetative cells of *B. subtilis* under certain conditions.

Recently, several papers and a review (23) have been published concerning the relationship between heat sensitivity and the occurrence of single strand scissions in DNA molecules as a consequence of heating, in some strains of *Escherichia coli* (24-30), *Salmonella typhimurium* (31,32), and *Streptococcus faecalis* (33). But it is still uncertain whether the single strand scissions of DNA molecules in vegetative cells are dependent on either the direct action of elevated temperature, or the action by nucleases released or activated by mild heating.

In this chapter, experiments were made on the degradation of

single stranded DNA by mild heating and its mechanism in the vegetative cells of *B. subtilis*.

## 2. MATERIALS AND METHODS

### Organism

*B. subtilis* 168Tt (thy<sup>-</sup>, trp<sup>-</sup>) was used throughout this work.

### Preparation of nonlabeled and labeled vegetative cells

Logarithmic-phase culture of *B. subtilis* was inoculated to 10 ml of the Demain's medium (75) supplemented with 20 µg/ml of tryptophan, 5 µg/ml of thymine and 5 µCi of [methyl-<sup>14</sup>C] thymidine (43.5 mCi/mmol : New England Nuclear). The cultures were grown at 37°C to an optimal density (660 nm) of 0.6. The cells were then harvested and washed 3 times with M/15 phosphate buffer (pH 7.2). Nonlabeled vegetative cells were grown in the same medium without radioisotope and were prepared by the same method used for labeled vegetative cells.

### Heat treatment and counting of the survivals

Washed cells were resuspended in M/15 phosphate buffer (pH 7.2). The suspensions were incubated at 50°C for various periods and then immediately cooled in an ice bath. The survivors of vegetative cells were counted as described in Chapter III Section 1.

### Preparation of protoplasts from vegetative cells and extraction of

### DNA

Heated and non-heated cells were converted to protoplasts by the

modification of the method of Weibull (102). The cells were incubated at 37°C for 15 min in lysozyme solution (500 µg/ml) containing 7 % sucrose and 10 mM EDTA. After centrifugation, the protoplasts were suspended in distilled water.

#### Sedimentation analysis of $^{14}\text{C}$ labeled DNA in alkaline sucrose density gradients

Zone sedimentation of DNA was performed by the method of McGrath and Williams(104). For sedimentation, a 4.6 ml linear concentration gradient of 5 - 20 % (w/v) alkaline sucrose (pH 12.0) was used. A 0.1 ml portion of 0.5 N NaOH containing 2 % SDS and a 0.2 ml portion of ruptured vegetative protoplasts were layered on the top of the sucrose gradient by using a screwpipette. The gradients were centrifuged at 30.000 rpm for 90 min at 20°C in a Hitachi RPS-40T2 rotor. After centrifugation, the contents were collected from bottom in fractions of 0.15 ml on the disks of filter paper. The disks were dried and counted in toluene system in a liquid scintillation counter.

### 3. RESULTS

#### Heat sensitivity of vegetative cells of *B. subtilis*

The thermal death curves of *B. subtilis* vegetative cells at different temperatures are given in Fig. 11. Vegetative cells of *B. subtilis* are more sensitive to heat than spores. By heating at 90°C

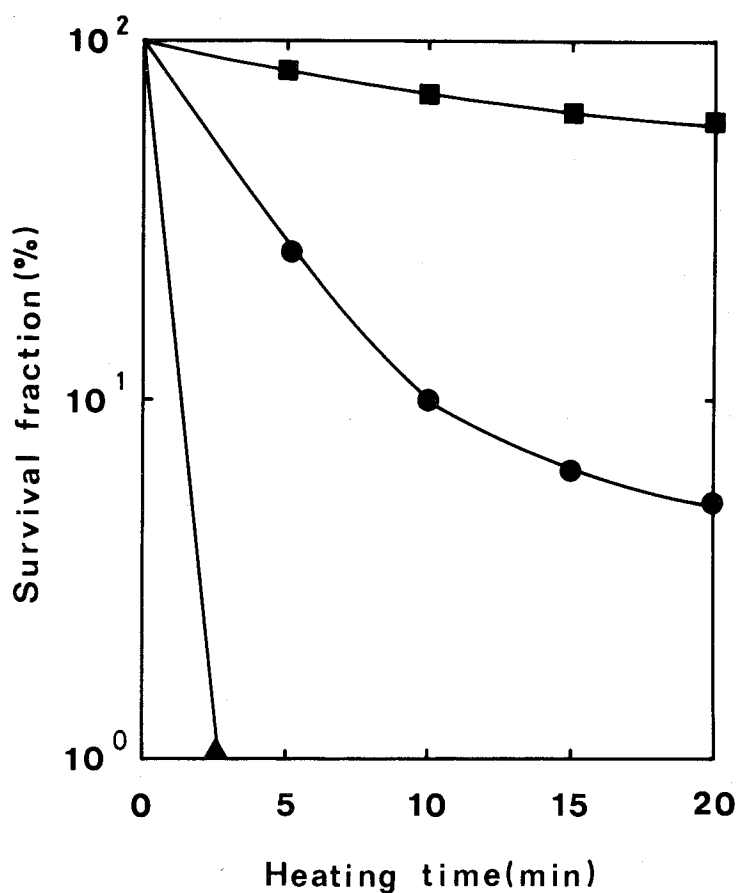


Fig. 11. Heat-survival curves of *B. subtilis* vegetative cells. The cells at logarithmic growth phase were used. Symbols : ■—■, heated at 46°C ; ●—●, heated at 50°C ; ▲—▲, heated at 90°C.

for 10 min all the vegetative cells died. After incubation at 50°C for 10 and 20 min, about 10 and 5 %, respectively, of the cells remained viable ; longer incubation times further reduced survival fractions.



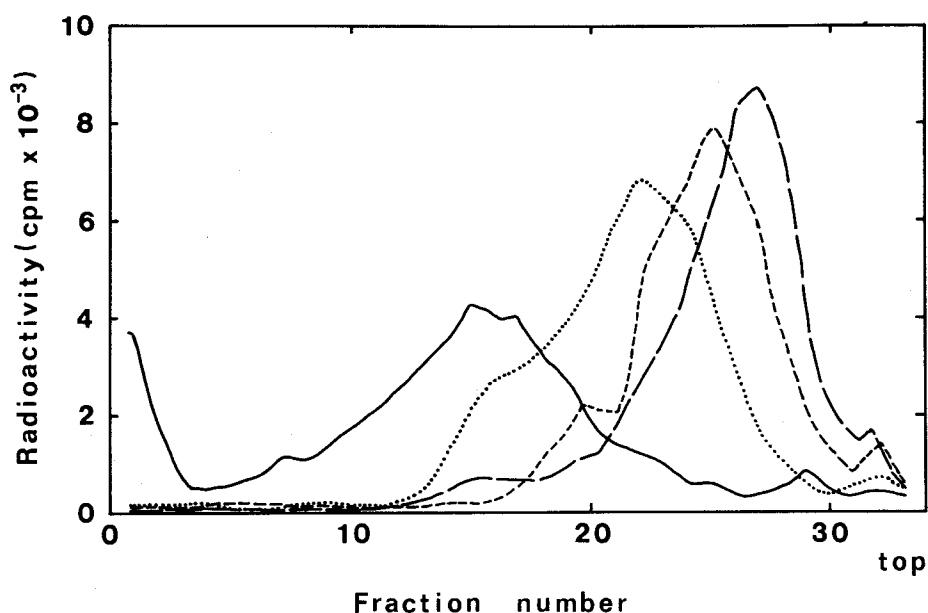


Fig. 12. Effect of heat treatment at 50°C on sedimentation patterns in alkaline sucrose density gradients of DNA from vegetative cells of *B. subtilis* grown on Demain's medium.

Symbols : —, not heated ; ·····, heated at 50°C for 10 min ; ---, heated at 50°C for 20 min ; — · —, heated at 50°C for 30 min.

#### Sedimentation analysis of DNA of heated vegetative cells

Figure 12 shows the sedimentation profiles for single strand DNA isolated from *B. subtilis* vegetative cells after heated at 50°C for 10, 20, and 30 min. The sedimentation distance of DNA was decreased in accordance with the decreasing of viability caused by mild heating. The mild heating either directly or indirectly gave rise to DNA single strand breakages.

Comparing with mild heating (50°C), vegetative cells heated at

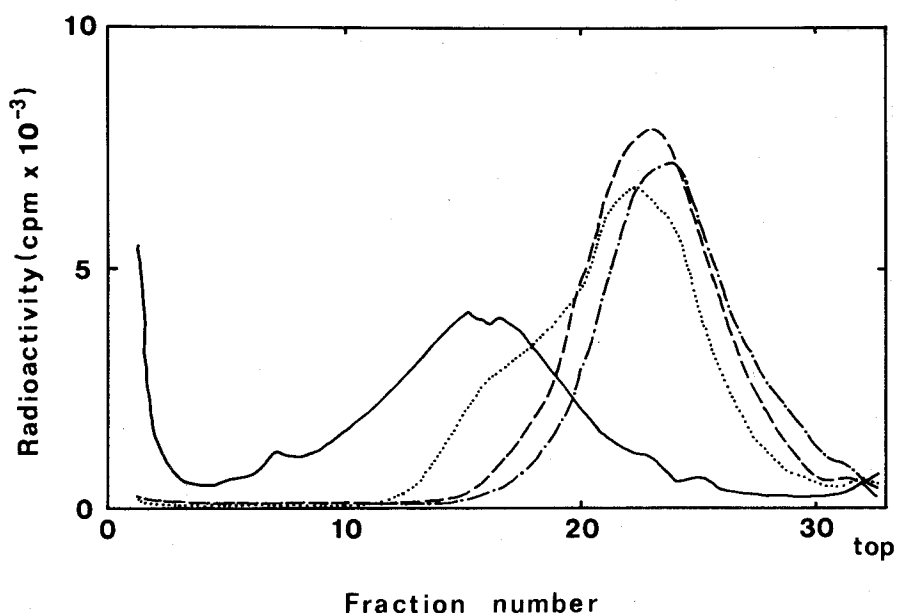


Fig. 13. Effect of heat treatment at different temperatures on sedimentation patterns in alkaline sucrose density gradients of DNA from vegetative cells of *B. subtilis* grown on Demain's medium.

Symbols : —, not heated ; ..... , heated at 50°C for 10 min ; ---, heated at 90°C for 10 min ; —.—, heated at 90°C for 20 min.

90°C for 10 or 20 min were subjected to sedimentation analysis.

Figure 13 shows that the sedimentation rate of DNA was decreased by heating at 90°C for 10 min or 20 min, however, the positions of the peaks in DNA from cells heated at 50°C for 10 min, 90°C for 10 min, and 90°C for 20 min were nearly same.

Figure 14 also shows that the sedimentation rate of DNA was decreased by heating at 46°C for 20 or 30 min as well as 50°C for 10 min.

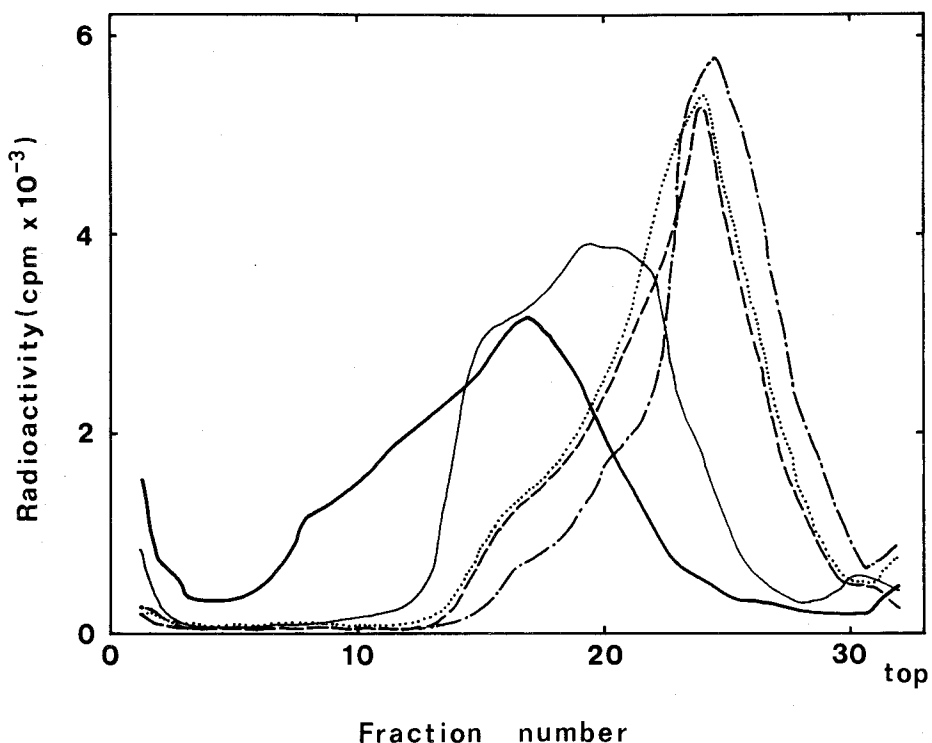


Fig. 14. Effect of mild heating at 46°C on sedimentation patterns in alkaline sucrose gradients of DNA from vegetative cells of *B. subtilis* grown on Demain's medium.

Symbols : —, not heated ; ·····, heated at 50°C for 10 min ; —, heated at 46°C for 10 min ; ---, heated at 46°C for 20 min ; —·—, heated at 46°C for 30 min.

When vegetative cells which had been heated at 70°C for 10 or 20 min were subjected to sedimentation analysis, DNA in the cells was sedimented at almost the same position with that heated at 50°C for 10 min (data not shown). However, in the case of vegetative cells grown on Schaeffer's medium to the middle log phase and heated at 50°C for 10 or 30 min, single strand scissions of DNA were not apparent.

These results indicate that the occurrence of single strand scissions of DNA in vegetative cells by heat treatment is dependent on both the growth stage and the physiological state of *B. subtilis* and has no relation to the heating temperature or the heating time. These data also suggest that the mild heating probably causes indirectly DNA strand breakage in vegetative cells.

#### Effect of p-CMB on DNA breakage of heated vegetative cells

It is considered that single strand breaks of DNA after heating at 50°C could be the result of nuclease action and not of the direct action of heat. In order to ascertain this hypothesis, the effect of p-chloromercuribenzoate (p-CMB) on sedimentation patterns in alkaline sucrose density gradients of DNA from vegetative cells of *B. subtilis* was studied.

Figure 15 A shows that the single strand scissions of DNA took place in vegetative cells when the cells were preincubated for 10 min at 37°C in phosphate buffer (0.067 M, pH 7.2) and then heated at 50°C for 10 or 30 min. However, when cells were preincubated for 10 min at 37°C in phosphate buffer (0.067 M, pH 7.2) containing p-CMB at a concentration of 1 mM and then heated at 50°C for 10 or 30 min, single strand scissions of DNA scarcely occurred (Fig. 15 B). These data strongly suggest that p-CMB inhibited endonuclease activity, which is probably released or activated by mild heating.

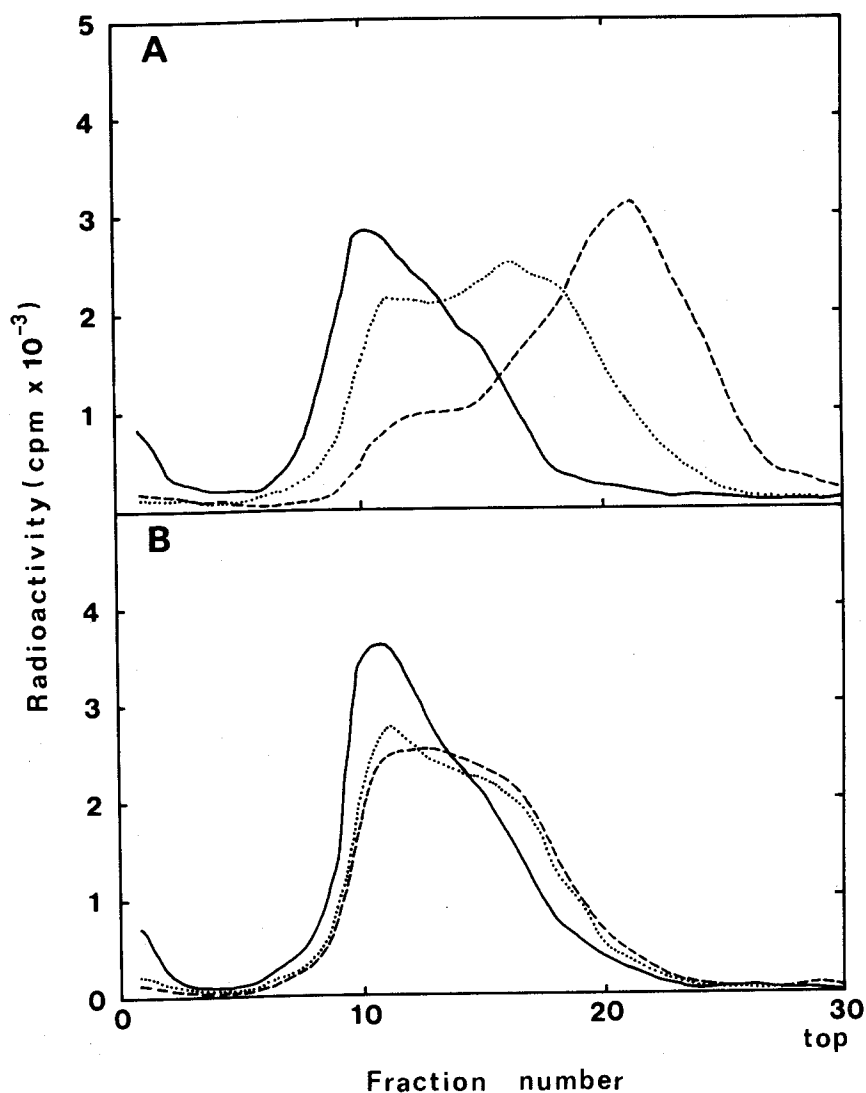


Fig. 15. Effect of p-CMB on sedimentation patterns in alkaline sucrose density gradients of DNA from vegetative cells of *B. subtilis* grown on Demain's medium.

A : — not heated (-p-CMB) ; ..... , heated at 50°C for 10 min (-p-CMB) ; --- , heated at 50°C for 30 min (-p-CMB) B : — , not heated (+p-CMB) ; ..... , heated at 50°C for 10 min (+p-CMB) ; --- , heated at 50°C for 30 min (+p-CMB).

#### 4. DISCUSSION

It has been reported that the heat treatment causes damages of many cellular components, such as cell wall, membrane, protein, RNA, and DNA, of bacterial cells (18-22) and that the injured cells were sensitive to some plating media and sometimes they were repaired in suitable media (104-114). Bridges et al. (24,25) demonstrated for the first time that after incubation of *E. coli* for similarly to several minutes at 52°C the single strand scissions of DNA occurred, similarly to the treatment by ionizing radiations. They proposed that the production of single strand breaks in DNA by mild heating may be the primary lethal event in the thermal inactivation of mesophilic bacteria.

In Chapter III Section 1, it was made clear that DNA breakages in heated-spores increased with an decrease in pH of heating buffer and scarcely occurred at neutral pH. However, in vegetative cells the single strand scissions of DNA were produced by mild heating at 50 °C for 10 min. Furthermore, in vegetative cells, DNA breakages were not dependent on the heating temperature or the heating time, but on both the growth stage and the physiological state of the cells.

Since heating of DNA at 52°C does not result in denaturation or strand breakage *in vitro*, and since the breakage depends on the cells being in the logarithmic phase of growth, Woodcock and Grigg (27) postulated that the DNA breakage was enzymatic.

DNA breakage in vegetative cells by mild heating is substantially reduced and scarcely occurred by adding enzyme inhibitor, p-CMB added before heating. Grecz and Bhatarakamol (30) reported that p-CMB inhibited the DNA breaks of *E. coli* by mild heating and a mutant of *E. coli* deficient in apurinic acid endonuclease yielded substantially fewer DNA breaks than wild type cells or cells of ligase deficient strain.

Therefore, it can be said that, in vegetative cells grown on nutrient-poor media, single strand DNA molecules are probably degraded by endonuclease which is activated or released by mild heating.

## 5. SUMMARY

Alkaline sucrose gradient sedimentation studies reveal that mild heating at 50°C caused the single strand scissions of DNA molecules in vegetative cells of *B. subtilis*. The DNA breakage was produced by heating at 46 or 90°C as well as at 50°C. The DNA breakages in vegetative cells were not dependent on the heating temperature and the heating time. Since the DNA breakages were inhibited by p-CMB, it is thought that the single strand scissions of DNA in vegetative cells are due to the activity of endonuclease, which is probably released or activated by mild heating.

## Chapter IV

### REPAIR OF DAMAGED DNA IN HEATED *B. SUBTILIS* SPORES DURING GERMINATION

#### 1. INTRODUCTION

In Chapter III Section 1, the author clarified that when treated at the lower pH and the higher temperature, the more the spores were inactivated and the single strand scissions of DNA in spores increased with decreasing pH in heating. However, DNA breakage in spores scarcely occurred by heating at 90°C for 30 min at pH 7.0. Therefore, it was thought that the single strand scissions of DNA in spores were dependent on depurination and the subsequent alkaline treatment.

AS DNA in spores is the most important target of  $\gamma$ -ray or UV, it is thought that DNA in irradiated spores is subjected to various injuries and most of the damages are repaired during germination. In fact, it has been shown that DNA damages caused by radiations and by chemical agents were repaired *in vivo* (9,11). However, as to DNA in spores, such studies were very few, because of the difficulty to extract DNA from spores in the natural state. It was shown that radiation-induced single strand scissions of DNA in spores of *B. subtilis* were rejoined during postirradiation incubation, and the rejoining during germination of the spores in nutrient medium occurred without detectable DNA synthesis (115).



In the present chapter, it was investigated whether or not DNA injuries of spores induced by acid-heating are repaired during germination, and whether or not the repair of DNA injury during germination requires of DNA synthesis.

## 2. MATERIALS AND METHODS

### Organism

*B. subtilis* 168Tt (thy<sup>-</sup>,trp<sup>-</sup>) was used throughout this work.

### Preparation of nonlabeled and <sup>3</sup>H-labeled spores

Nonlabeled spores were prepared by growing *B. subtilis* at 37°C in Schaeffer's medium (74) according to the method described in Chapter III.

To obtain the spores labeled with <sup>3</sup>H-thymidine the same medium supplemented with 2.5 µCi/ml [methyl-<sup>3</sup>H] thymidine (58 Ci/mmol : New England Nuclear) was used as described in Chapter III.

### Acid heat treatment

Nonlabeled and labeled spores were heated as described in Chapter III.

### Extraction of DNA from spores and germination spores

The DNA of heated and unheated <sup>3</sup>H-labeled spores, which were not germinated, was extracted with the method described in Chapter III.

In order to extract DNA from spores incubated after heating, heated spores were centrifuged and resuspended in Schaeffer's medium

to germinate. After incubation at 37°C for desired periods, the germinated spores were harvested and treated with 1 mg/ml lysozyme in 0.01 M Tris-HCl (pH 8.0), 0.01 M EDTA-4Na, 0.2 % Brij-58 and 10 % sucrose at 37°C for 30 min and centrifuged. The pellet was suspended and solubilized slowly in 2 % sodium dodecyl sulfate in 0.1 N NaOH at 37°C for 30 min and then DNA was extracted.

#### Sedimentation analysis of $^3\text{H}$ -labeled DNA in alkaline sucrose density gradient

Extracted DNA was layered on linear 5 - 20 % alkaline sucrose density gradient (pH 12.0) and centrifuged at 40,000 rpm for 2 h in a Beckman SW.50.1 rotor. The contents were fractionated and radioactivity was counted as described in Chapter III.

#### Measurement of DNA synthesis during germination

Heated and unheated spores were incubated at 37°C in 10 ml of Schaeffer's medium supplemented with 10  $\mu\text{Ci}$  of [methyl- $^3\text{H}$ ] thymidine (58 Ci/mmol : New England Nuclear). Incorporation of  $^3\text{H}$ -labeled thymidine into the trichloroacetic acid insoluble fraction was taken as a measure of the synthesis of DNA. The bacterial concentration in the culture was about  $10^8$  spores per ml. At intervals, 0.2 ml samples were taken in duplicate from the incubation culture. After the samples were suspended in 2 ml of 10 % cold trichloroacetic acid for 10 min, the samples were collected on a Millipore filter (0.22  $\mu\text{m}$  pore size) and washed two times with ethanol ; they were then dried and counted in a scintillation counter.

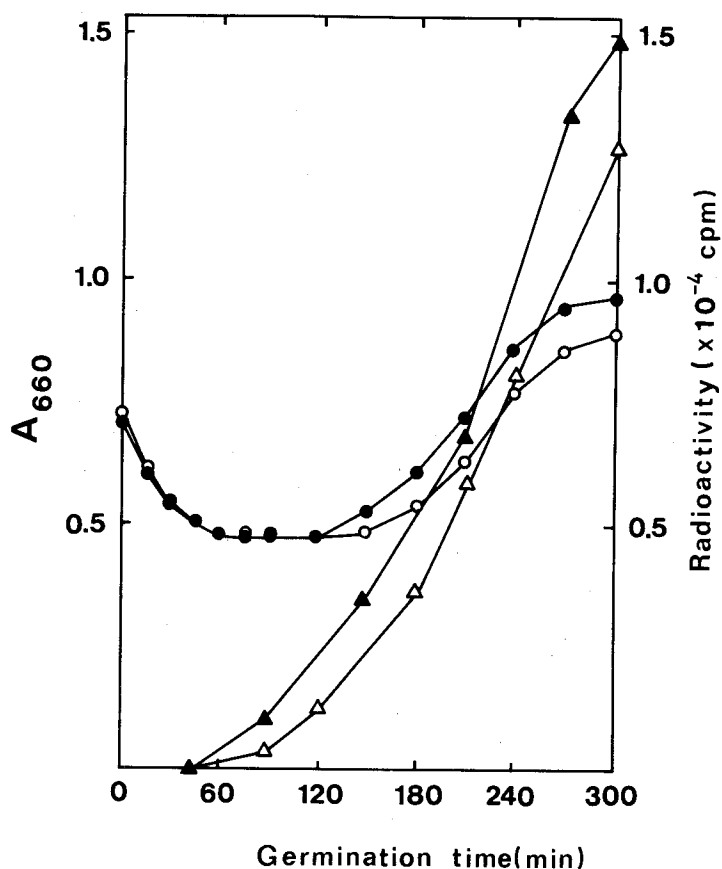


Fig. 16. DNA synthesis of *B. subtilis* spores during postheating incubation in germination medium.

▲—▲,  $^3\text{H}$ -thymidine incorporated into unheated spores ; △—△,  $^3\text{H}$ -thymidine incorporated into spores heated at  $70^\circ\text{C}$  for 20 min at pH 5.0 ; ●—●, absorbance at 660 nm of unheated spores ; ○—○, absorbance at 660 nm of spores heated at  $70^\circ\text{C}$  for 20 min at pH 5.0.

### 3. RESULTS

#### DNA synthesis during germination of heated spores

In Chapter III Section 1, it was found that the heat resistance of spores decreased with decreasing pH. When the spores were heated at 70°C, the survival fraction after 20 min heating at pH 7.0 was about 90 %, however at pH 5.0, the survival fraction after 20 min heating at 70°C was about 60 %. Furthermore, the single strand scissions of DNA in spores occurred by heating at 70°C for 20 min at pH 5.0, but not at pH 7.0. Therefore, in order to study the mechanism of DNA repair during germination, DNA synthesis during postheating incubation of acid-heated spores was measured by estimative <sup>3</sup>H-thymidine incorporation into acid insoluble materials. At the same time the change in turbidity of the germination culture was measured. The result is shown in Fig. 16. The decrease in optical density at the initial stage of germination of acid-heated spores was similar to that of the spores without heating. However, the subsequent increase in optical density of heated spores was delayed for 30 min than that of unheated spores. The DNA synthesis of unheated spores was initiated after 60 min of incubation in germination medium. In spores which was heated at 70°C for 20 min at pH 5.0, DNA synthesis was initiated after 90 min incubation. In the case of the spores which were heated at 70°C for 30 min at pH 3.0, DNA synthesis in spores was not observed even after 5 h incubation in germination medium (data is not shown).

#### Rejoining of DNA single strand breaks during germination

As DNA synthesis of spores heated at 70°C for 20 min at pH 5.0 was initiated after 90 min of incubation in germination meidum, the

change in the molecular weight of single strand DNA from the heated spores was observed with the lapse of time in the post heating incubation. As shown in Fig. 17, the single strand scissions of DNA in spores incubated by acid-heating were scarcely restored after 1 h postheating incubation at 37°C. After 2 h postheating incubation, consistently, most of the DNA breakages were restored to the levels of DNA from nonheated spores. After 3 h postheating incubation, the single strand scissions of DNA were completely repaired.

These results show that in case that most of the heated spores are survival the single strand scissions of DNA in spores which occurred through depurination, are restored during germination.

However, the single strand scissions of DNA in spores heated at 70°C for 30 min at pH 3.0 (about 1 % survival) were hardly repaired even after 6 h incubation in germination medium (data is not shown).

#### 4. DISCUSSION

The primary objective of this chapter was to investigate the condition under which DNA injury in heated spores was repaired during germination after acid-heat treatment. For this purpose, <sup>3</sup>H-labeled DNA was extracted from *B. subtilis* spores which were germinated after acid-heating (70°C 20 min, pH 5.0) and the extent of single strand scissions of DNA molecules was measured by alkaline sucrose gradient centrifugation.

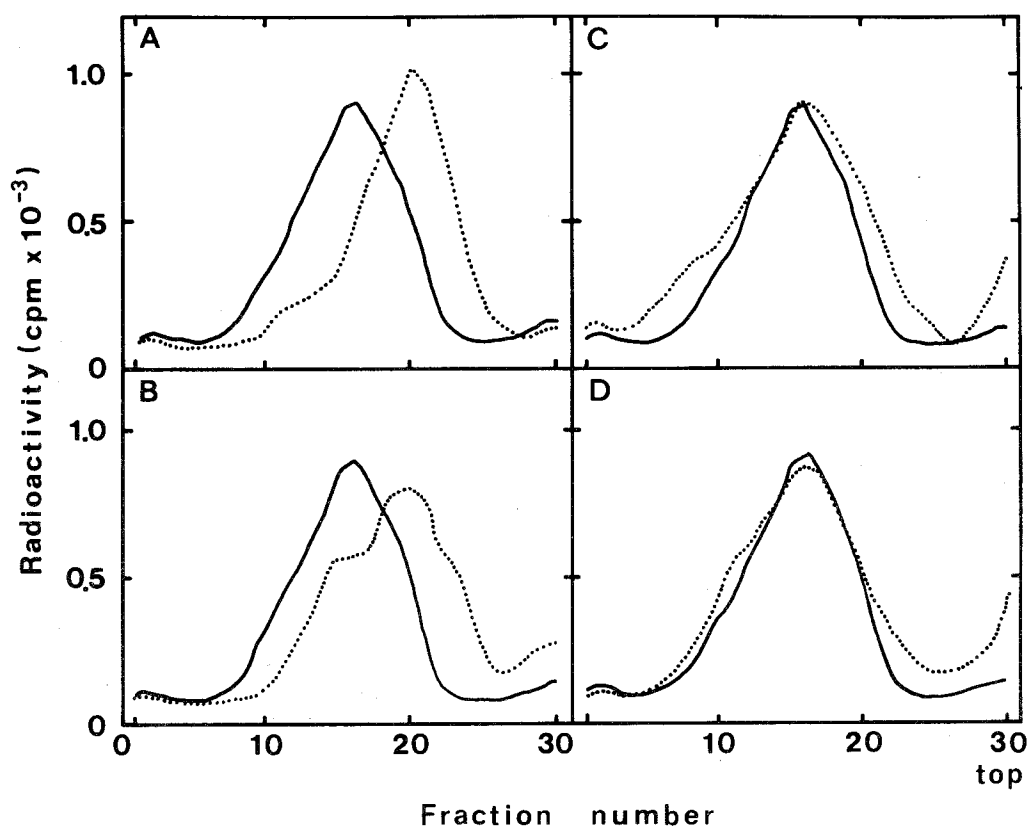


Fig. 17. Alkaline sucrose density gradient profiles for repair of acid-heated DNA in spores of *B. subtilis*.

$^3\text{H}$ -labeled spores were heated at  $70^\circ\text{C}$  for 20 min (pH 5.0) and immediately incubated in germination medium at  $37^\circ\text{C}$ . At the indicated time,  $^3\text{H}$ -labeled DNA was extracted and studied by sedimentation analysis.

The direction of sedimentation is from right to left.

Symbols : A, —, non-heating; ·····, just after heating. B, —, 1 h incubation of non-heated spores ; ·····, 1 h incubation of heated spores. C, —, 2 h incubation of non-heated spores ; ·····, 2 h incubation of heated spores. D, —, 3 h incubation of non-heated spores ; ·····, 3 h incubation of heated spores.

In Chapter III Section 1, it was found that single strand scissions of DNA in spores heated at 70°C at pH 7.0 did not occur even after heating for 30 min, but at pH 5.0 DNA breakage increased in parallel to the increase in heating time. When the heated spores were germinated in Schaeffer's medium, the single strand scissions of DNA were repaired in 2 h after germination. Furthermore, DNA synthesis of acid-heated spores was initiated after 90 min of incubation. When heated at 70°C for 30 min at pH 3.0 (about 1 % survival), however, DNA synthesis in spores was not initiated after 5 h of incubation, and the single strand scissions of DNA in spores were hardly recovered during 6 h incubation. It was found that the postheating recovery time increased with increasing the DNA breaks in spores.

Terano et al. (115) reported that the single strand breaks of DNA in spores which were irradiated with 190 K rad (8 % survival dose) were rejoined during postirradiation incubation and the rejoining in spores which were germinated in nutrient medium occurred in the absence of detectable DNA synthesis.

Therefore, it is thought that the DNA damages and its recovery system in spores which were treated with  $\gamma$ -ray and heat were different in quality each other.

## 5. SUMMARY

When DNA in spores of *B. subtilis* was injured by heat treatment

at 70°C for 20 min at pH 5.0, at which the survivals did not decrease greatly, the molecular weight of single stranded DNA was restored to the level of non-heated spores during germination after heating. The rejoining of DNA in spores during germination in nutrient medium was terminated after about 2 h postheating incubation.



## Chapter V

### DEPURINATION OF PURIFIED DNA BY HEATING

#### 1. INTRODUCTION

As described in Chapter III Section 1, it was found that the single strand scissions of DNA in heated spores increased with decreasing pH. And the scissions hardly occurred at neutral pH. Therefore, it was thought that acid-heat treatment produced the apurinic sites in DNA in spores and then DNA was broken at the apurinic sites by the subsequent alkaline treatment.

In the present chapter, the conditions which are responsible for the depurination in purified DNA have been studied.

#### 2. MATERIALS AND METHODS

##### Organism

*B. subtilis* 168Tt (thy<sup>-</sup>, trp<sup>-</sup>) and *B. subtilis* (ade<sup>-</sup>, thr<sup>-</sup>, hisA, lys<sup>-</sup>, trp<sup>-</sup>, met<sup>-</sup>) which was kindly provided by H. Tanooka were used.

##### Sedimentation analysis of purified DNA from vegetative cells in alkaline sucrose density gradient

Non labeled DNA of *B. subtilis* 168Tt vegetative cells was purified according to the method of Marmur (116). A 20 µl aliquot of 1 M NaOH

was added to 200  $\mu$ l of depurinated DNA which was heated at various temperatures and various pH values. Then the solution was incubated at 37°C for 15 min. This treatment hydrolyzes a phosphoester bond near the apurinic site (117). The DNA was layered on linear 5 - 20 % alkaline sucrose density gradient (pH 12.0) and centrifuged at 40,000 rpm for 4.5 h at 5°C in a Beckman SW.50.1 rotor. The gradients were analyzed in an ISCO density gradient fractionator (Model 185) and UV-absorbance monitor (Model UA-5).

#### Preparation of purine-labeled DNA

A purine-requiring mutant of *B. subtilis* ( $ade^-$ ,  $thr^-$ ,  $hisA$ ,  $lys^-$ ,  $trp^-$ ,  $met^-$ ) was grown in Schaeffer liquid nutrient broth (74) supplemented with 250  $\mu$ Ci/l of [ $8-^3H$ ] guanine sulfate (15 Ci/mmol ; The Radiochemical center, Amersham) at 37°C, and was harvested at the late logarithmic growth phase. The DNA was extracted according to the method of Marmur (116), dissolved in SSC solution (0.15 M NaCl/ 0.015 M sodium citrate, pH 7.0) and stored at -20°C.

#### Analysis of depurination

One ml of purine labeled DNA was heated at various pH (pH 3, 5, 7) and chilled. Calf thymus DNA in SSC (400  $\mu$ g/200  $\mu$ l) and 200  $\mu$ l of 12.5 % perchloric acid were added to depurinated DNA. After being kept at 0°C for 10 min, the mixtures were centrifuged at 13,000 x g for 10 min, and the radioactivity of the supernatants was determined in toluene-Triton scintillation fluid in a liquid scintillation counter.

### 3. RESULTS

#### Temperature and pH dependence of depurination

It is generally said that the apurinic/apyrimidinic sites in DNA are labile to the alkaline treatment resulting chain breaks at the 3'-side of apurinic sugar moieties (118) and that these apurinic/apyrimidinic sites are introduced by acid-heat treatment.

In order to ascertain the condition of depurination of purified DNA, the DNA solutions of which pH values were adjusted to 3.0, 5.0, and 7.0 were heated at 50, 60, 70, 80, and 90°C. After the alkaline treatment to break the apurinic sites molecular weights of the DNA were analyzed by alkaline sucrose density gradient centrifugation. The DNA heated at pH 7.0 for 30 min below 90°C, were scarcely depurinated (Fig. 18), although the single strand scissions of DNA in vegetative cells occurred by mild heating at 50°C. On the other hand, at pH 5.0 and 3.0 the sedimentation distance of heated DNA decreased with the increase of temperature and of heating time (Fig. 19).

Consequently, depurination sites increases with decrease in pH value and temperature.

#### Release of $^3\text{H}$ -labeled purines from DNA

In order to measure directly the release of purines from double-stranded DNA in solution, it is necessary to heat the DNA solutions for relatively long periods of time. The [ $^3\text{H}$ ] purine-labeled *B. subtilis* DNA was heated in SSC at pH 3, 5, or 7 and 70 or 80°C for 0.5

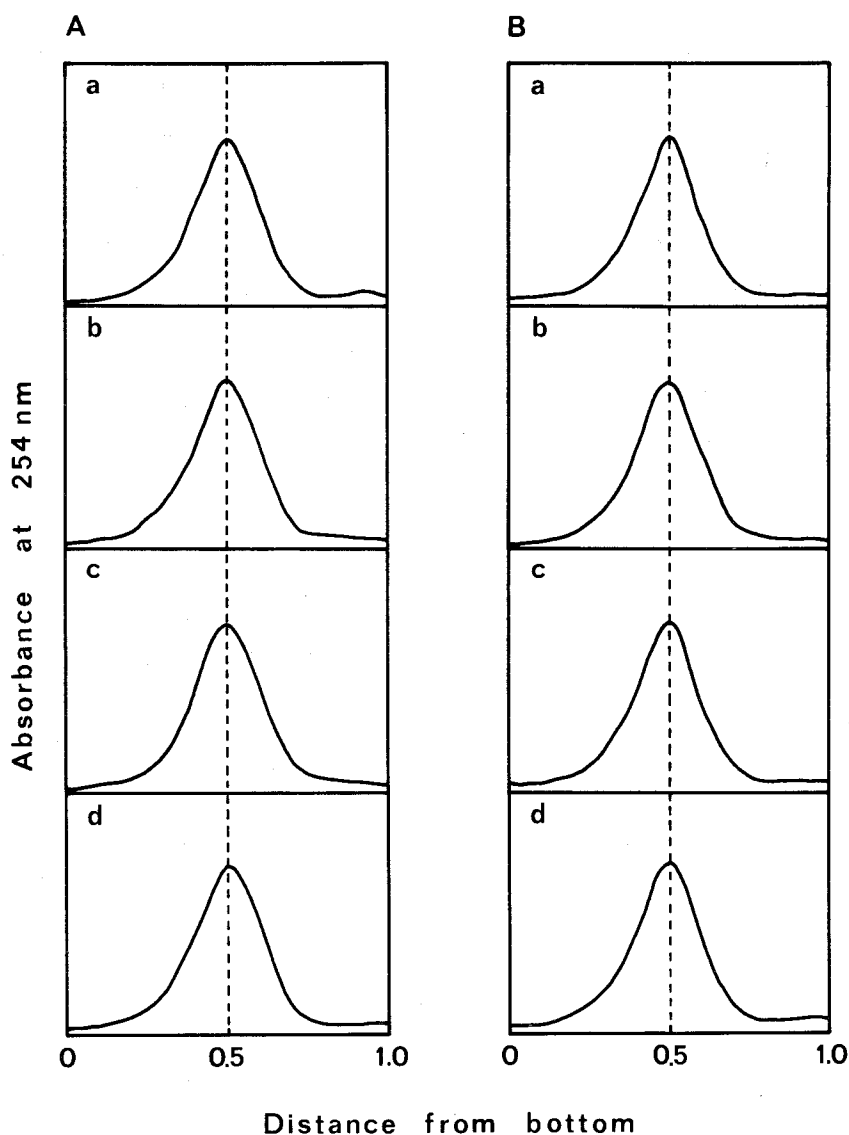


Fig. 18. Effect of temperature on depurination of purified DNA at neutral pH. Following heating at pH 7.0 purified DNA was treated with alkali and studied by sedimentation analysis. The direction of sedimentation is from right to left. A : a, non-heated ; b, heated at 50°C for 10 min ; c, heated at 50°C for 20 min ; d, heated at 50°C for 30 min. B : a, non-heated ; b, heated at 70°C for 30 min ; c, heated at 80°C for 30 min ; d, heated at 90°C for 30 min.

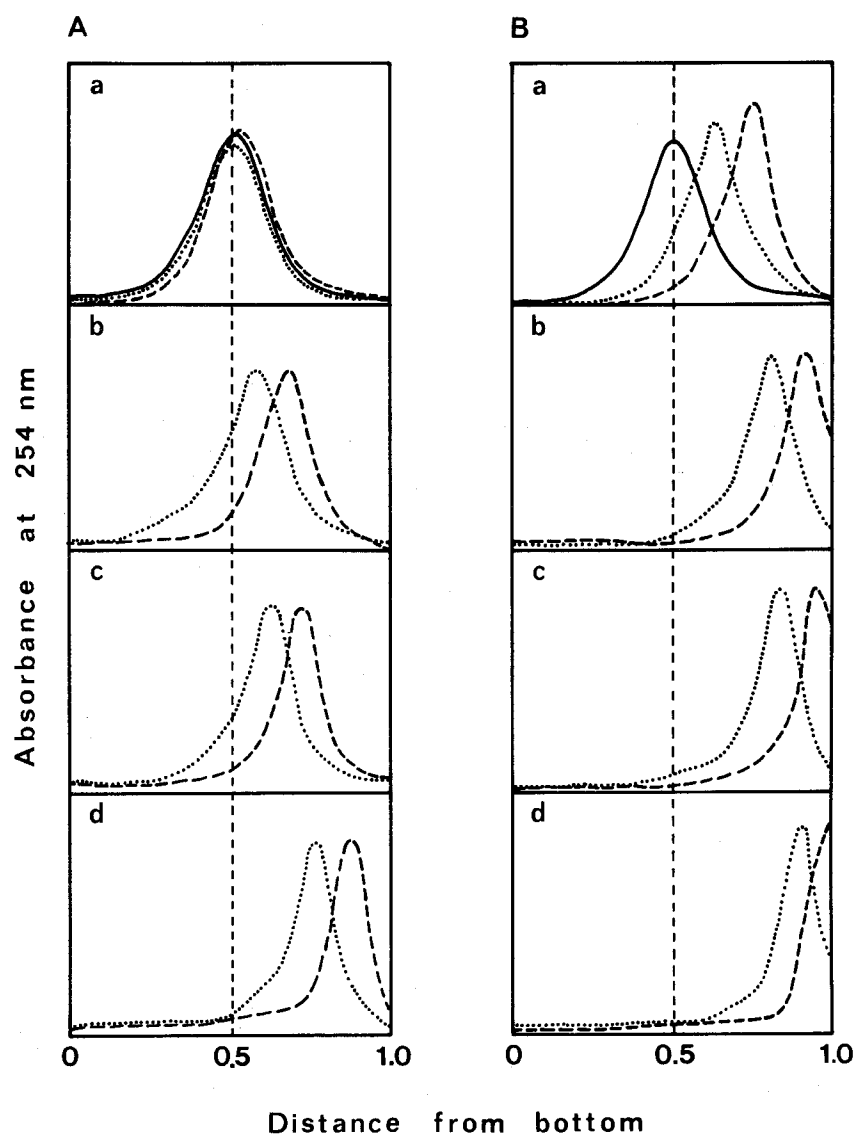


Fig. 19. Effect of temperature on depurination of purified DNA at pH 5.0 (A) and at pH 3.0 (B).

Following heating at pH 5.0 and at pH 3.0 purified DNA was treated with alkali and studied by sedimentation analysis. The direction of sedimentation is from right to left. Symbols : a, 60°C ; b, 70°C ; c, 80°C ; d, 90°C. —, non-heated ; ..... , heated for 10 min ; ---, heated for 30 min.

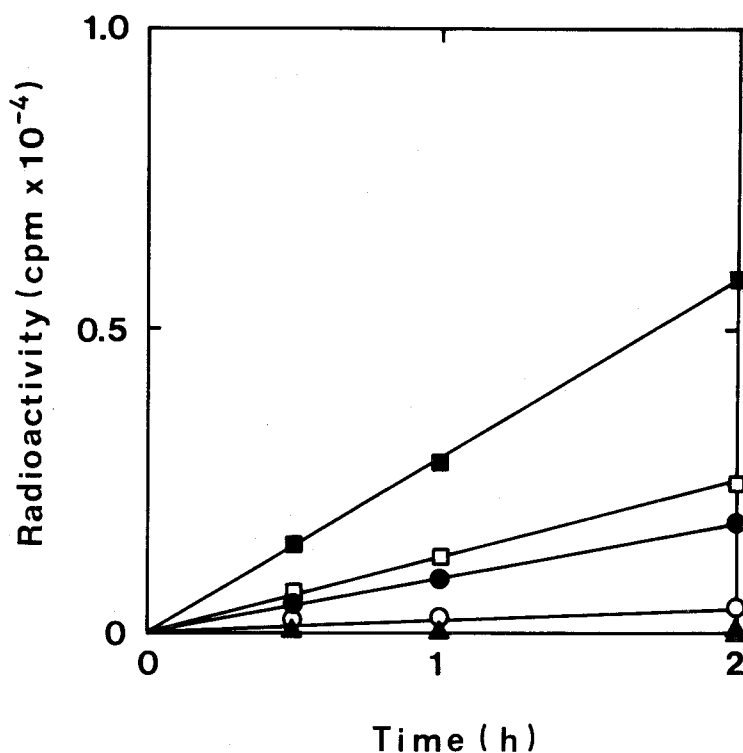


Fig. 20. Temperature and pH dependences of depurination.

[<sup>3</sup>H]purine labeled *B. subtilis* DNA was heated at 70°C and 80°C at pH 3 to 7 and radioactive material released from DNA in acid-soluble form was measured.

Symbols :  $\triangle-\triangle$ , heated at 70°C at pH 7.0 ;  $\circ-\circ$ , heated at 70°C at pH 5.0 ;  $\square-\square$ , heated at 70°C at pH 3.0 ;  $\blacktriangle-\blacktriangle$ , heated at 80°C at pH 7.0 ;  $\bullet-\bullet$ , heated at 80°C at pH 5.0 ;  $\blacksquare-\blacksquare$ , heated at 80°C at pH 3.0.

to 2 h. Under these conditions, radioactive material was found to be released from DNA in acid-soluble fraction (Fig. 20). At pH 5, the degree of depurination was much slower than at pH 3. At neutral pH, however, depurination hardly occurred at 70°C or 80°C for 2 h.

At pH 7, depurination occurred more rapidly at higher temperature.

These results also show that depurination site increases with decrease in pH value and increase in temperature.

The results of acid-heat induced DNA injury of spores, described in Chapter III Section 1, correspond to that of depurination of DNA heated at low pH *in vitro*.

It was concluded, therefore, that DNA in spores of *B. subtilis* was depurinated by acid-heat treatment, but at neutral pH depurination of DNA in spores hardly occurred in the temperature range between 60 and 90°C.

#### 4. DISCUSSION

In the previous Chapter, it was shown that the single strand scissions of DNA in spores increased with decrease in pH and increase in temperature. However, at neutral pH DNA breaks hardly occurred.

Greer and Zamenhof (38) demonstrated that purine bases were released in detectable quantities from calf thymus DNA at temperatures near the  $T_m$ . Lindahl and Nyberg (47) reported that in a  $Mg^{2+}$ -containing buffer of physiological ionic strength, the rate constant for depurination of DNA was  $4 \times 10^{-9}$ /sec at 70°C and pH 7.4 and the rate of depurination at pH 5.0 was higher than at pH 7.4. Therefore, it was thought that depurination likely occurred first by the acid-heat treatment of spores and DNA was broken at apurinic sites by the sub-

sequent alkaline treatment.

In order to study the pH and temperature dependence of depurination, the sedimentation analysis and direct measurement of released [ $^3\text{H}$ ]-labeled purines from DNA were made. The results indicate that the depurination site increased with decrease in pH and increase in temperature.

The results shown in Chapter III that the lower pH and the higher temperature, the more the DNA in spores was broken correspond to the results obtained in the present Chapter.

It was concluded, therefore, that acid-heat treatment induced the apurinic sites in DNA in spores.

## 5. SUMMARY

In order to study the temperature and pH dependence of *in vitro* depurination of DNA, purified DNA after being heated at various pH and temperature was analyzed by the sedimentation analysis. Furthermore, released [ $^3\text{H}$ ] purines from DNA by acid-heating were directly measured. It was found that the number of depurination site increased with decrease in pH value and increase in temperature.



## Chapter VI

### PURIFICATION AND CHARACTERIZATION OF APURINIC/APYRIMIDINIC ENDODEOXY-RIBONUCLEASE SPECIFIC TO HEAT INDUCED APURINIC SITES IN DNA OF *B.*

#### *SUBTILIS*

##### 1. INTRODUCTION

Under various conditions, nucleoside bases are released from cellular DNA through the hydrolysis of N-glycosidic bonds, thereby leaving apurinic or apyrimidinic sites (119,120). Nucleoside bases are released by spontaneous hydrolysis, acid hydrolysis, heat treatment (38,47), and  $\gamma$ -irradiation (121,122). Uracil which appeared in DNA either by misincorporation of dUMP instead of dTMP residues (123) or by deamination of cytosine by heat (124), bisulfite (125), or nitrous acid (126), is removed by a uracil-DNA glycosylase (48). In addition, some modified bases in DNA which had been treated with alkylating agents like methylmethane sulfonate were subjected to spontaneous or enzymatic release (127).

As shown in Chapter III and V, DNA bases were released by *in vivo* and *in vitro* acid-heat treatments. Therefore, the repair of apurinic or apyrimidinic sites is probably essential for the maintenance of cellular DNA.

The endonucleases specific to apurinic sites in DNA have been

isolated from *E. coli* (51-53), and *H. influenzae* (54), *M. luteus* (55,56), *B. subtilis* (57), and *B. stearothermophilus* (58). These endonucleases have been studied in relation to the DNA injuries induced by alkylating agents, X-ray irradiation, ultraviolet irradiation, or carcinogenic substances and their repair system.

It was found in the previous chapter that acid-heat treatment induced apurinic sites in DNA of spores and the damaged DNA was repaired during germination.

In this chapter a new APendodeoxyribonuclease (Apurinic/apyrimidinic endodeoxyribonuclease) which acts specifically on the heat injured DNA was isolated from *B. subtilis* and characterized.

## 2. MATERIALS AND METHODS

### Preparation of substrates

*B. subtilis* 168Tt (thy<sup>-</sup>, trp<sup>-</sup>) was grown at 37°C in Schaeffer liquid nutrient broth (74) containing 0.5 µCi/ml [methyl-<sup>3</sup>H] thymidine (50 Ci/mmol ; The Radiochemical Center, Amersham), and was harvested at the late logarithmic growth phase. The DNA was extracted according to the method of Marmur (116), dissolved in SSC solution (0.15 M NaCl / 0.015 M sodium citrate, pH 7.0) and stored at -20°C.

Alkylated [<sup>3</sup>H] DNA and alkylated-heated[<sup>3</sup>H] DNA were prepared according to the method of Verly and Rassart(52). To the [<sup>3</sup>H]DNA dissolved in SSC were added 2 volumes of 1 M sodium phosphate buffer,

pH 7.0 / methylmethane sulfonate to a final concentration of 0.3 M and the solution was incubated at 37°C for 1 h. The mixture was cooled on ice and dialyzed three times at 4°C against SSC (alkylated [<sup>3</sup>H]DNA). Under these conditions, the DNA contained about 550 methyl groups/10<sup>6</sup> daltons (128). The alkylated [<sup>3</sup>H]DNA in SSC was heated at 50°C for 6 h and dialyzed three times at 4°C against SSC containing 0.1 M MgCl<sub>2</sub>. By this treatment about 160 alkylated purines / 10<sup>6</sup> daltons of DNA were lost from alkylated DNA. Approximately 40 % of the alkylated-heated DNA was recovered as acid-soluble fraction after treatment with NaOH which caused a strand break near each apurinic site. The depurinated (=alkylated heated) [<sup>3</sup>H]DNA thus obtained was stored at -20°C.

In order to obtain single strand DNA or depurinated single strand DNA, native DNA or a alkylated-heated DNA was dialyzed against 90 % formamide, 5 mM potassium phosphate (pH 6.8) and was incubated at 37°C for 2 h. After chilling, DNA solutions were again dialyzed against 1 % formaldehyde, 5 mM sodium phosphate (pH 6.8), and were applied to hydroxyapatite column to isolate the single strand DNA by use of method of Miyazawa and Thomas (129).

The DNA which contains small number of apurinic sites was prepared by heating at 60°C for 30 min in SSC (pH 3.5).

Heated DNA was reduced by the modified method described by Hadi and Goldthwait (130) in order to protect the apurinic sites from alkaline treatment.

For the end group analysis, the radioactive reduction of depurinated DNA was prepared according to the modified method of Gossard and Verly (131). Sodium boro- $[\text{}^3\text{H}]$ -hydride (260 mCi/mmol : New England Nuclear) was dissolved in distilled water to a final concentration of 10  $\mu\text{g}/\mu\text{l}$ . Solution of depurinated DNA (200  $\mu\text{g}/\text{ml}$ ) was dialyzed against 0.01 M sodium borate buffer, pH 9.8. Portions of 50, 25, and 25  $\mu\text{l}$  of sodium boro- $[\text{}^3\text{H}]$ -hydride solution were successively added and the mixture was left at room temperature for 1 h ; non-labeled  $\text{NaBH}_4$  (15 mg/200  $\mu\text{l}$ ) in 0.01 M NaOH was finally added. After 30 min the excess hydride was destroyed with 0.2 ml of 1 M sodium acetate buffer, pH 5.0. When the hydrogen release finished, the solution was dialyzed six times against 2 l SSC containing 0.01 M  $\text{MgCl}_2$  (pH 7.0) at 4°C for a week.

#### Enzyme assay

Ten  $\mu\text{l}$  of alkylated-heated DNA containing 0.1 M  $\text{MgCl}_2$  labeled with [methyl- $^3\text{H}$ ] thymidine (0.67  $\mu\text{g}$ , 11,000 cpm) in SSC (pH 7.0) and 80  $\mu\text{l}$  of buffer A (50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, 10 % glycerol, pH8.0) were mixed with 10  $\mu\text{l}$  of enzyme solution in buffer A. After incubation at 37°C for 15 min, the tubes were chilled and 200  $\mu\text{l}$  of SSC containing 400  $\mu\text{g}$  of calf thymus DNA and 200  $\mu\text{l}$  of 12.5 % perchloric acid were successively added to each tube to stop the enzyme reaction. After being kept at 0°C for 15 min, the mixtures were centrifuged at 13,000 x g for 10 min, and the radioactivity of the supernatants was determined in toluene-TritonX-100

scintillation fluid in a liquid scintillation counter. One unit of enzyme activity was defined as the amount of enzyme activity necessary to release 10 % acid-soluble radioactivity of the substrate.

#### Sedimentation coefficient determination

The sedimentation coefficient and molecular weight of APendodeoxyribonuclease were determined by glycerol gradient centrifugation by the method of Martin and Ames (132). Linear 10 to 30 % (v/v) glycerol gradient in buffer A was used. Centrifugation was performed at 4°C for 24 h at 38,000 rpm in a Beckman SW.50.1. rotor. The gradients were fractionated with ISCO density gradient fractionator and UV-absorbance monitor (Model UA-5). APendodeoxyribonuclease activity in each fraction was assayed.

#### Polyacrylamide gel electrophoresis

Electrophoresis was carried out with 7.5 % polyacrylamide gels as described by Gabriel (133). After electrophoresis at 4°C, one of the gels was stained with coomassie brilliant blue for detection of protein and another gel was cut in 3 mm slices. The slices were eluted over night with 0.2 ml of buffer A and APendodeoxyribonuclease activity was measured.

#### Sodium dodecyl sulfate gel electrophoresis

The method described by Weber and Osborn (134) was followed. Cytochrome c, chymotrypsinogen A, ovalbumin, and bovine serum albumin were used as marker proteins.

#### Sedimentation analysis of DNA in alkaline sucrose density gradient

DNA was layered on linear 5 - 20 % alkaline sucrose density gradient (pH 12.0) and centrifuged at 40.000 rpm for 4.5 h at 5°C in a Beckman SW.50.1 rotor. The gradients were analyzed in an ISCO density gradient fractionator and UV-absorbance monitor (Model UA-5).

#### Protein determination

Protein was determined by the method of Lowry et al. (135) by using bovine serum albumin as a standard.

#### Enzymes

*E. coli* alkaline phosphatase and snake venom phosphodiesterase were bought from Sigma Chemical Co. Spleen phosphodiesterase and polynucleotide 5'-hydroxyl-kinase were purchased from Boehringer Mannheim Co.

### 3. EXPERIMENTS AND RESULTS

#### Activities of the crude extract

*B. subtilis* 168Tt (thy<sup>-</sup>, trp<sup>-</sup>) was grown in nutrient broth (0.5 % peptone and 0.5 % beef extract) at 37°C and was harvested at the late logarithmic growth phase. The vegetative cells were suspended in buffer A and disrupted with French press. The suspension was centrifuged at 16,000 x g for 30 min. The supernatant is referred to as the crude extract.

Fig. 21. gives the acid-soluble radioactivity formed as a function of the amount of enzyme ; the acid-soluble radioactivity increased

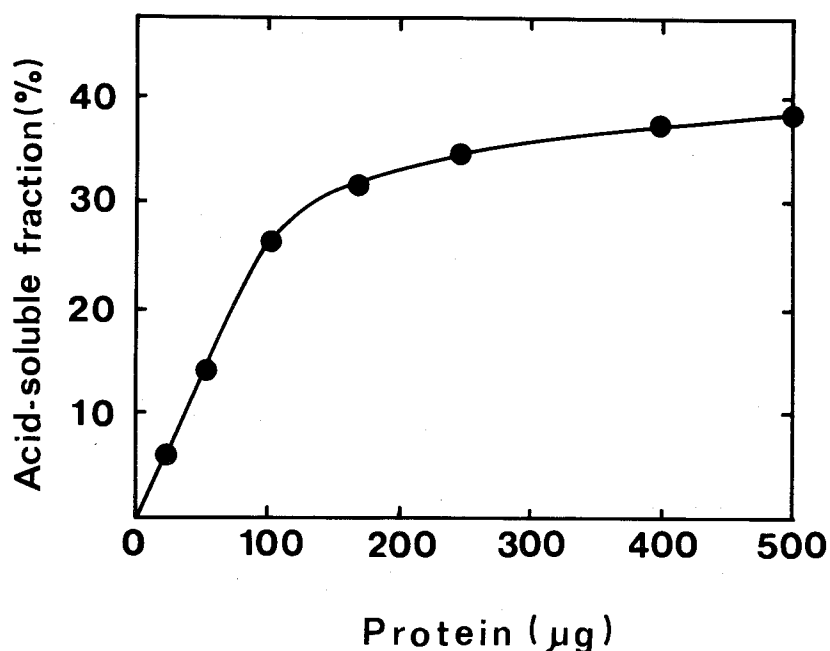


Fig. 21. Acid-soluble radioactivity produced from depurinated [ $^3\text{H}$ ] DNA as a function of the enzyme concentration.

To 50  $\mu\text{l}$  of alkylated-depurinated DNA, was added 50  $\mu\text{l}$  of buffer A containing increasing amounts of the crude extract. After 15 min at 37°C, the acid-soluble radioactivity was measured. The acid-soluble radioactivity (in per cent of the substrate radioactivity) is given as a function of the amount of enzyme (expressed in micrograms of protein).

rapidly first and approached a maximum value of 40 %. This is the acid-soluble radioactivity obtained after an incubation with NaOH instead of enzyme. Fig. 21 shows that, as long as the acid-soluble radioactivity remains under 25 %, it is approximately proportional to the amount of enzyme. In all assays used to measure the amount of APendodeoxyribonuclease activity, the enzyme was diluted in

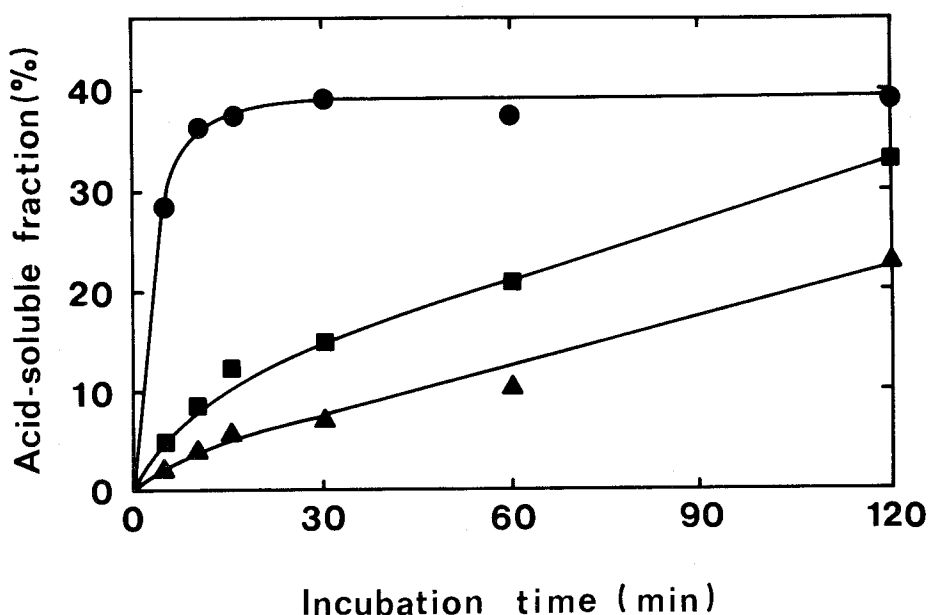


Fig. 22. Action of the crude extract of *B. subtilis* cells on native, alkylated, and alkylated-depurinated DNA.

Samples (100  $\mu$ l) of the crude extract were mixed with 100  $\mu$ l of [ $^3$ H] DNA, either native (▲), alkylated (■), or alkylated-depurinated (●) : the mixtures were incubated at 37°C and aliquots taken from 0 to 120 min to measure the acid-soluble radioactivity. The results are expressed as fractions of the substrate radioactivity (acid-soluble fraction).

buffer A so that the acid-soluble radioactivity liberated from depurinated [ $^3$ H]DNA fell below 25 %.

Crude extract was mixed with  $^3$ H-labeled native DNA, alkylated DNA, and depurinated DNA, respectively, and incubated at 37°C ; aliquots were taken after 0 to 120 min to measure the acid-soluble radioactivity. Fig. 22 shows that the acid-soluble fraction in



depurinated DNA increased rapidly during the first 15 min at 37°C reaching 38 % of the input radioactivity, but in native DNA only 5 % of the total radioactivity was made acid-soluble for 15 min.

Alkylated DNA was hydrolyzed more slowly than depurinated DNA but faster than native DNA.

#### Purification of enzyme

*B. subtilis* 168Tt (thy<sup>-</sup>, trp<sup>-</sup>) was grown in nutrient broth at 37°C by use of jar fermenter. The cells were harvested at late exponential phase by centrifugation, washed with cold TM buffer (0.01 M Tris-HCl, 0.06 M NH<sub>4</sub>Cl, 0.01 M Mg-acetate, and 0.07 M 2-mercaptoethanol, pH 7.5), and stored at -20°C. The frozen cell paste (216 g) was thawed and suspended in 800 ml of buffer A. Cells were disrupted by four 10 min periods with intermittent cooling with a Tomy ultrasonic disruptor UR200P at 0°C. The cell debris was removed by centrifugation at 17,000 x g for 30 min (Preparation I). To the supernatant, 3.2 % streptomycin sulfate in buffer A was added slowly to a final concentration of 0.8 %. The suspension was centrifuged at 17,000 x g for 20 min. The supernatant fraction was treated with solid ammonium sulfate at 4°C to reach 50 % saturation ; after centrifugation, the supernatant was brought to 80 % saturation. After centrifugation, the sediment was resolved in buffer A and dialyzed against the same buffer (Preparation II).

A column (3.6 x 20 cm) of DEAE-cellulose (Whatman) was equilibrated with buffer A at 4°C. Preparation II was applied and the

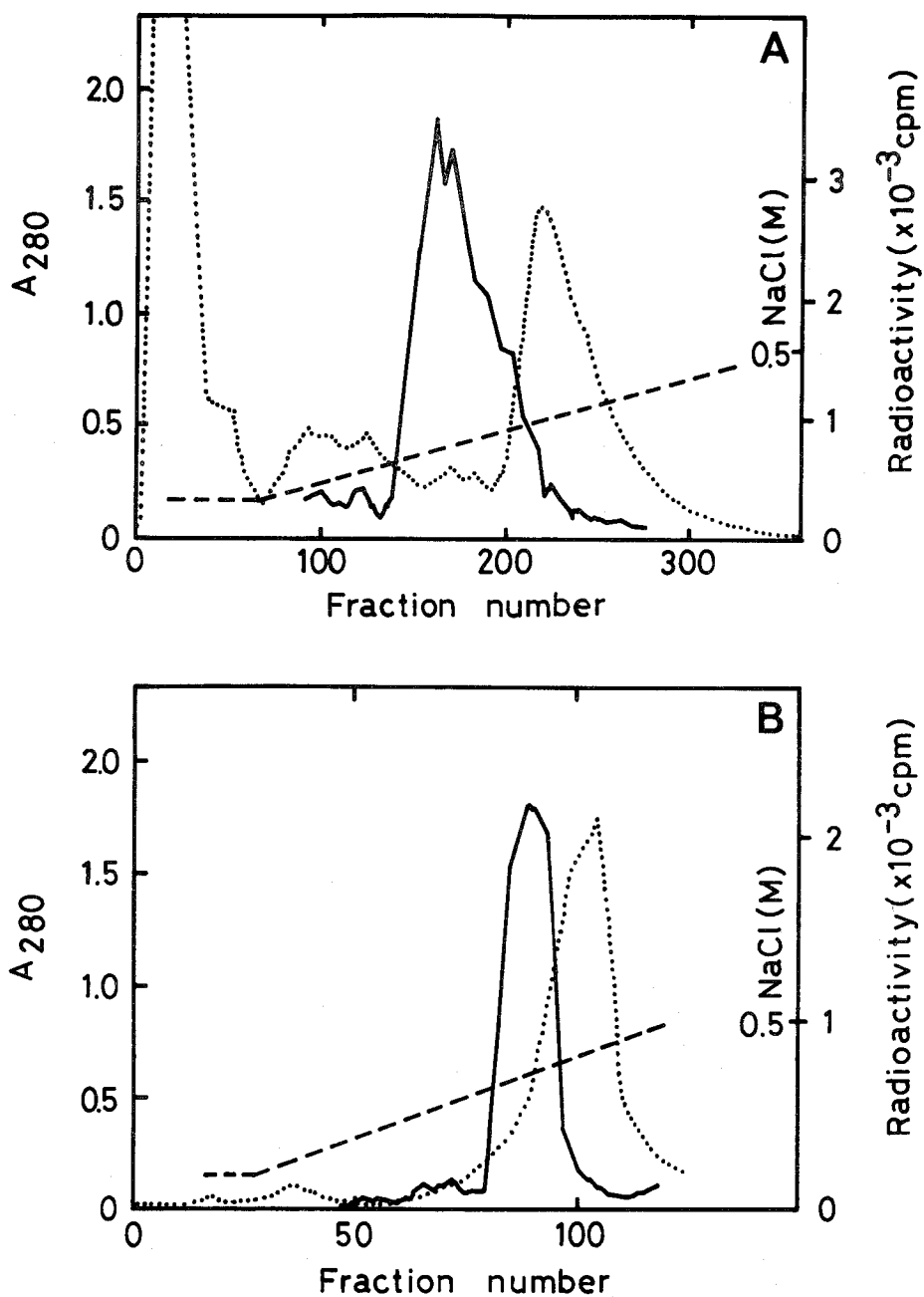


Fig. 23. Chromatographic purification of APendodeoxyribonuclease. Symbols : —, enzyme activity on depurinated DNA ; ·····, absorbance at 280 nm ; ---, concentration of NaCl (M). A, DEAE-cellulose ; B, DEAE-Sephadex A-50.

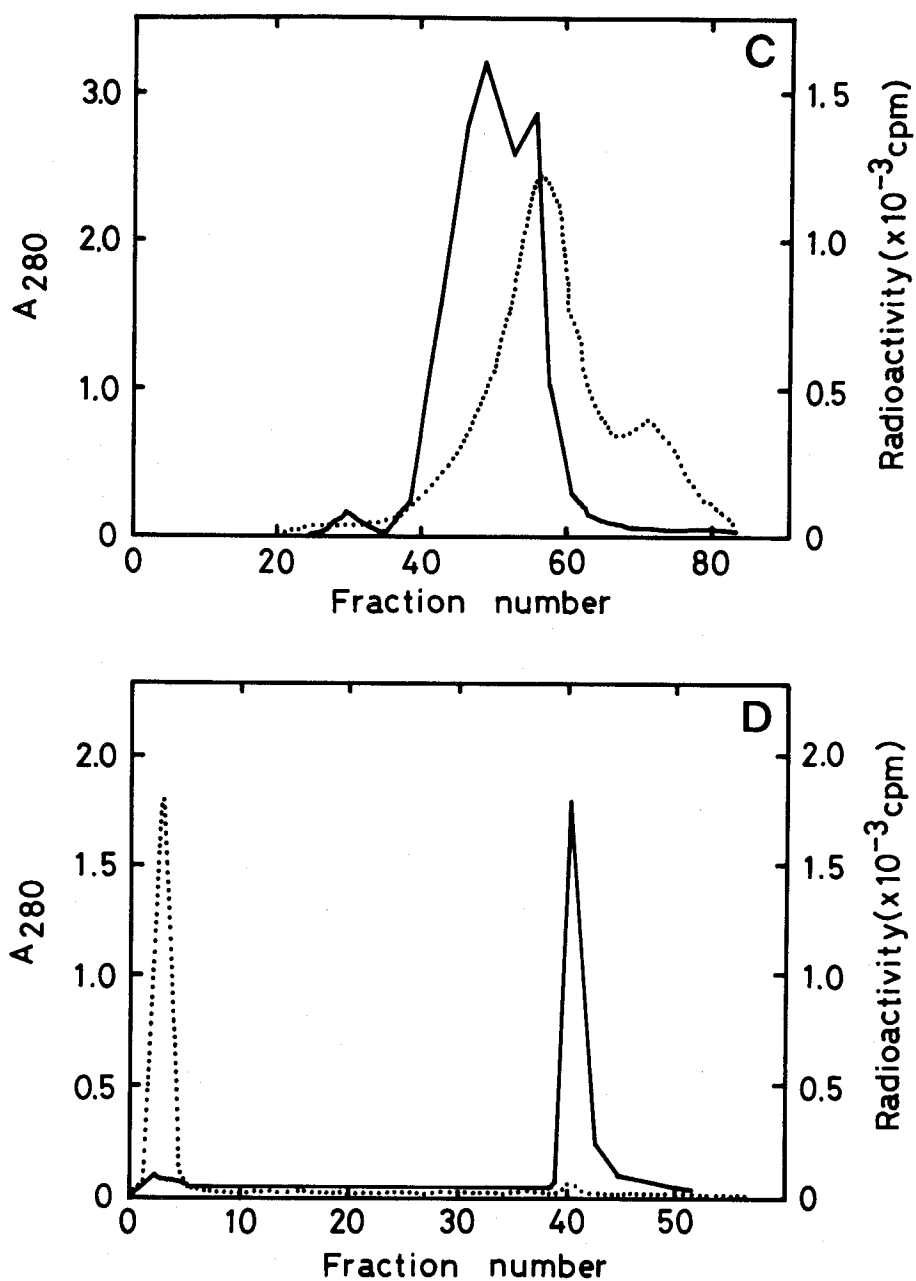


Fig. 23. Chromatographic purification of APendodeoxyribonuclease. Symbols : ———, enzyme activity on depurinated DNA ; ..... , absorbance at 280 nm. C, Sephadex G-200 ; D, DNA-cellulose.

column was washed with 200 ml of buffer A and 500 ml of the same buffer containing 0.1 M NaCl. Six liters of a 0.1 M to 0.5 M NaCl linear gradient in buffer A was eluted at rate of 120 ml/h ; fractions of 20 ml were collected. Figure 23 A shows that the enzyme was eluted between 0.25 and 0.32 M NaCl. Fractions 145 to 200 were pooled and concentrated to 160 ml with a Millipore ultrafiltration apparatus with a PSAC filter and then dialyzed against buffer A (Preparation III).

A column (2 x 25 cm) of DEAE-Sephadex A-50 (Pharmacia) was equilibrated with buffer A at 4°C. Preparation III was applied to the column at a flow rate of 40 ml/h. The column was washed with 100 ml of buffer A containing 0.1 M NaCl and eluted with 1 liter of a 0.1 M to 0.5 M NaCl linear gradient in buffer A ; fractions of 10 ml were collected. Figure 23 B shows that the enzyme was eluted between 0.32 and 0.41 M NaCl. Fractions 83 to 96 were pooled (Preparation IV).

Three ml of the concentrated Preparation IV were placed on a Sephadex G-200 (Pharmacia) column (2.8 x 40 cm) equilibrated with buffer A ; the elution was carried out with the same buffer at a rate of 12 ml/h, and 2.5 ml fractions were collected (Fig. 23 C). Fractions 41 to 50 were pooled (Preparation V).

DNA-cellulose was prepared according to the method of Litman (136). Preparation V was concentrated and applied to a DNA-cellulose (0.8 x 7 cm) equilibrated with buffer A. The column was washed with

Table 3. Purification of *B. subtilis* endonuclease for apurinic sites.

Preparation	Volume	Total protein	Endonuclease units	Specific activity
I Crude extract	838 ml	12402 mg	2022 x 10 <sup>3</sup> units	163 units/mg
II Ammonium sulfate	370	7918	1679	212
III DEAE-cellulose	157	184	1260	6850
IV DEAE-Sephadex	140	57.4	1095	19100
V Sephadex G-200	35	28.4	895	31500
VI DNA-cellulose	8	0.304	436	1430000

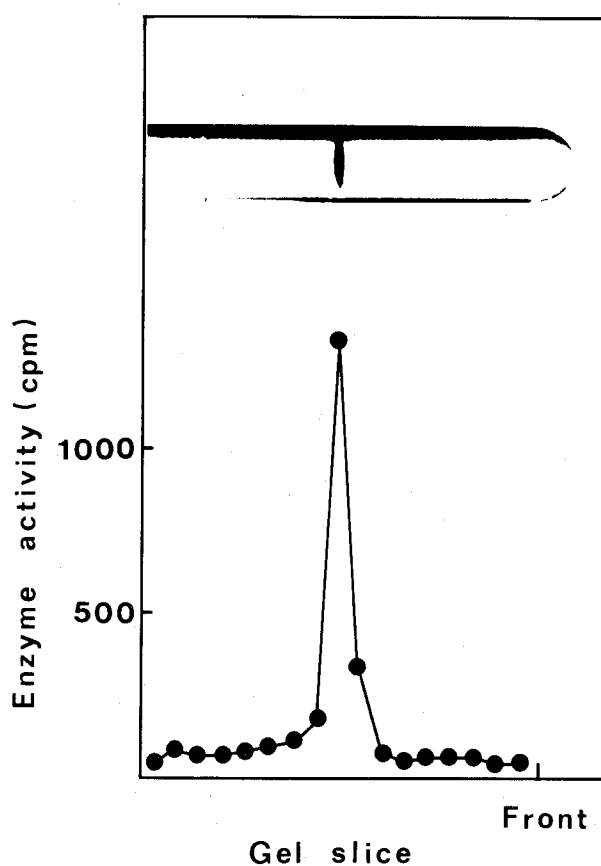


Fig. 24. Polyacrylamide gel electrophoresis of Preparation VI of APendodeoxyribonuclease.

Upper : a photograph of the gel stained with Commassie brilliant blue  
; lower : enzyme activity on alkylated-depurinated [<sup>3</sup>H]DNA.

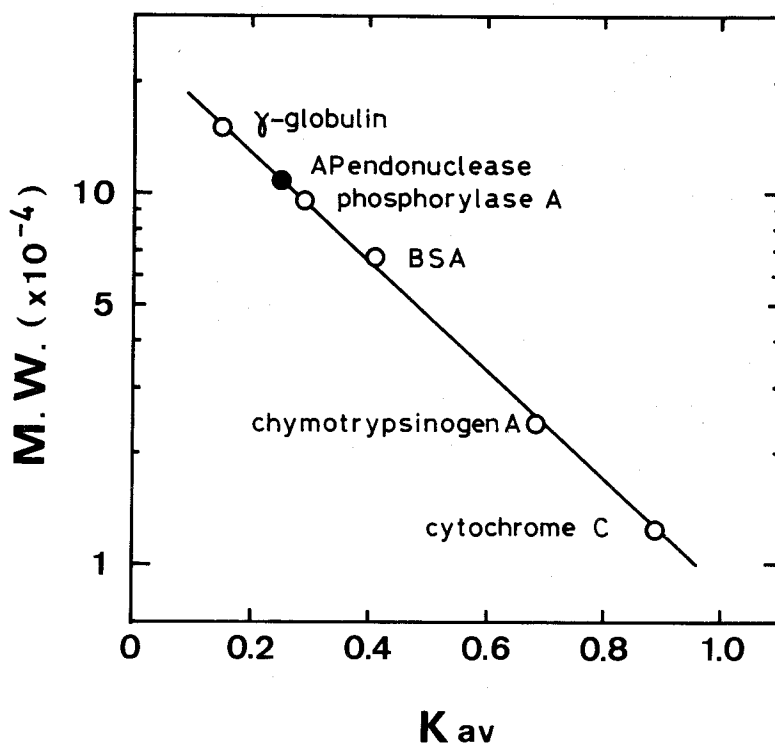


Fig. 25. Molecular weight determination of APendodeoxyribonuclease on Sephadex G-200.

The abbreviation and molecular weight (in parentheses) of standard proteins are :  $\gamma$ -globulin (156,000) ; phosphorylase A (96,000) ; bovine serum albumin(BSA) (68,000) ; chymotrypsinogen A (25,000) ; cytochrome c (12,000).

80 ml of the same buffer, and then eluted with 0.4 M NaCl in buffer A at a rate of 4 ml/h ; fractions of 2 ml were collected (Fig. 23 D). The fractions showing activity were pooled and dialyzed against the buffer A (Preparation VI), and the enzyme solution was kept at  $-20^{\circ}\text{C}$ .

These purification procedures are summarized in Table 3.

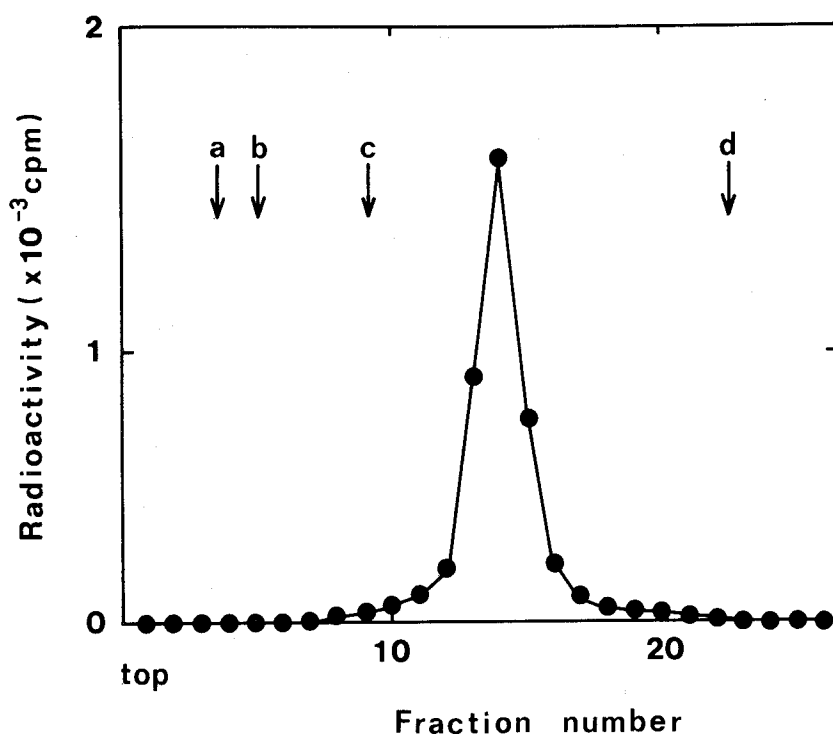


Fig. 26. Glycerol gradient centrifugation of APendodeoxyribonuclease. Parallel gradients were run with the four marker proteins as standards. The positions of the standards are given by arrows : (a) cytochrome c, (b) chymotrypsinogen A, (c) bovine serum albumin, (d) catalase.

About 8,800-fold purification was achieved with an overall yield of about 22 %.

The purity of the enzyme was determined by polyacrylamide gel electrophoresis. Gel electrophoresis revealed a single protein band which correspond to the APendodeoxyribonuclease activity (Fig. 24).

#### Properties of the enzyme

##### Molecular weight

The molecular weight of APendodeoxyribonuclease

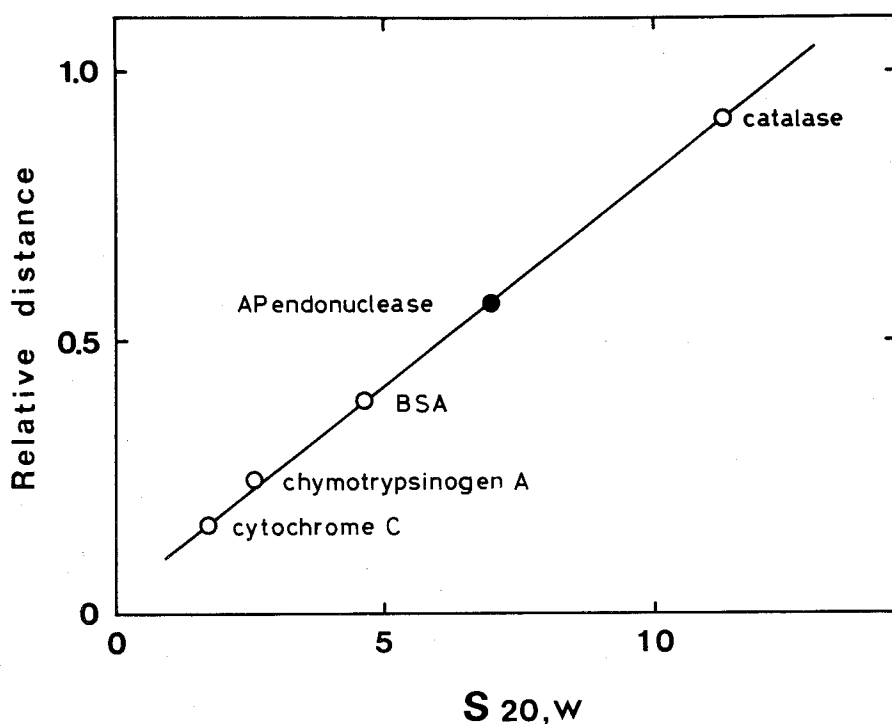


Fig. 27. Determination of molecular weight of APendodeoxyribonuclease by glycerol gradient centrifugation.

The sedimentation coefficient ( $S_{20,w}$ ) and molecular weight (in parentheses) of the standard proteins are : catalase from beef liver, 11.3 S (230,000) ; bovine serum albumin (BSA), 4.6 S (68,000) ; chymotrypsinogen A, 2.54 S (25,000) : chytochrome c, 1.84 S (12,000)

was determined by Sephadex G-200 column chromatography. The elution volumes of APendodeoxyribonuclease and reference proteins are plotted against the logarithms of their molecular weight (Fig. 25). From this plot, the molecular weight of APendodeoxyribonuclease was estimated to be 108,000.

Furthermore, in order to ascertain this result, the molecular



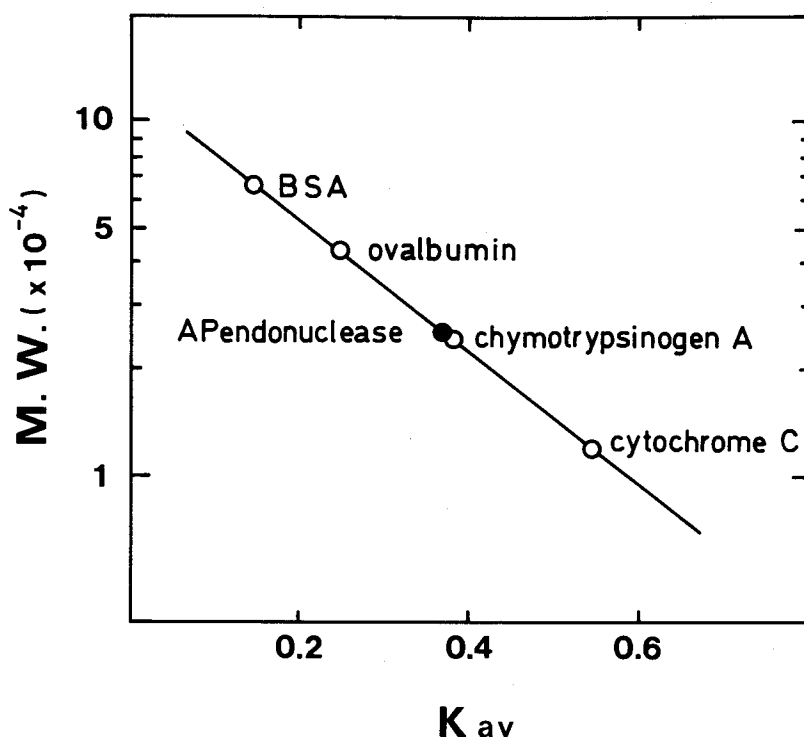


Fig. 28. Determination of molecular weight of denatured APendodeoxy-ribonuclease by SDS-polyacrylamide gel electrophoresis.

The abbreviation and molecular weight (in parentheses) of standard proteins are bovine serum albumin(BSA) (68,000) ; ovalbumin (43,000) ; chymotrypsinogen A (25,000) ; cytochrome c (12,000).

weight of APendodeoxyribonuclease was estimated by glycerol gradient centrifugation. The enzyme activity of Preparation VI migrated as a single peak in glycerol gradient centrifugation (Fig. 26). In comparison with the marker proteins, the sedimentation coefficient of APendodeoxyribonuclease was calculated to be 7 S (Fig. 27). The molecular weight of APendodeoxyribonuclease was estimated to be

105,000 daltons according to the method devised by Martin and Ames (132), assuming that the enzyme had a spherical shape.

Sodium dodecyl sulfate gel electrophoresis revealed that the purified enzyme (Preparation VI) was composed of a single protein.

Table 4. Effects of EDTA and  $Mg^{2+}$  on APendonuclease activity.

Ten  $\mu$ l of alkylated-depurinated DNA in SSC (pH 7.0) and 10  $\mu$ l of Preparation VI dialyzed against buffer B (0.05 M Tris-HCl, pH 8.0/0.1 mM 2-mercaptoethanol/10 % glycerol) were mixed with 80  $\mu$ l of buffer B ; EDTA or  $MgCl_2$  was added to reach the indicated concentration. After incubation at 37°C for 15 min, radioactivity in the acid-soluble fraction was measured.

Experiment	Addition	Concentration (mM)	Relative activity (%)
1 <sup>a</sup>	EDTA	0	100
		0.5	96
		1.0	102
		5.0	103
		10.0	65
		20.0	4 .
2 <sup>b</sup>	$Mg^{2+}$	0	10
		1.0	8
		2.0	13
		5.0	28
		10.0	55
		20.0	100
		40.0	91
		60.0	44
		80.0	30
		100.0	19

a, In the first experiment, all reaction mixtures contained 10 mM  $MgCl_2$ .

b, In the second experiment, EDTA was absent in all reaction mixtures.

The molecular weight of this protein was estimated to be about 26,000 by comparison with the electrophoretic mobilities of standard proteins (Fig. 28). Therefore, it is thought that this enzyme consists of four identical 26,000 daltons subunits.

#### Requirements for divalent cations

APendodeoxyribonuclease activity on depurinated [ $^3\text{H}$ ]DNA was measured as functions of the concentration of  $\text{Mg}^{2+}$  (Table 4). The purified enzyme absolutely required  $\text{Mg}^{2+}$  for its activity, and no activity was detected in the absence of  $\text{Mg}^{2+}$ . The optimum concentration of  $\text{MgCl}_2$  was 20 mM and the enzyme activity was inhibited at higher concentrations.

Table 5. Effects of  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$  on APendonuclease activity.

Ten  $\mu\text{l}$  of alkylated-depurinated DNA in SSC (pH 7.0) and 10  $\mu\text{l}$  of Preparation VI dialyzed against buffer B were mixed with 80  $\mu\text{l}$  of buffer B ;  $\text{MnCl}_2$  or  $\text{CaCl}_2$  was added to reach the indicated concentration. After incubation at  $37^\circ\text{C}$  for 15 min, radioactivity in the acid-soluble fraction was measured.

Addition	Concentration (mM)	Relative activity (%)
None	0	3
$\text{MnCl}_2$	1	6
	2	10
	5	22
	10	19
	20	8
$\text{CaCl}_2$	1	4
	2	4
	5	3
	10	3
	20	4

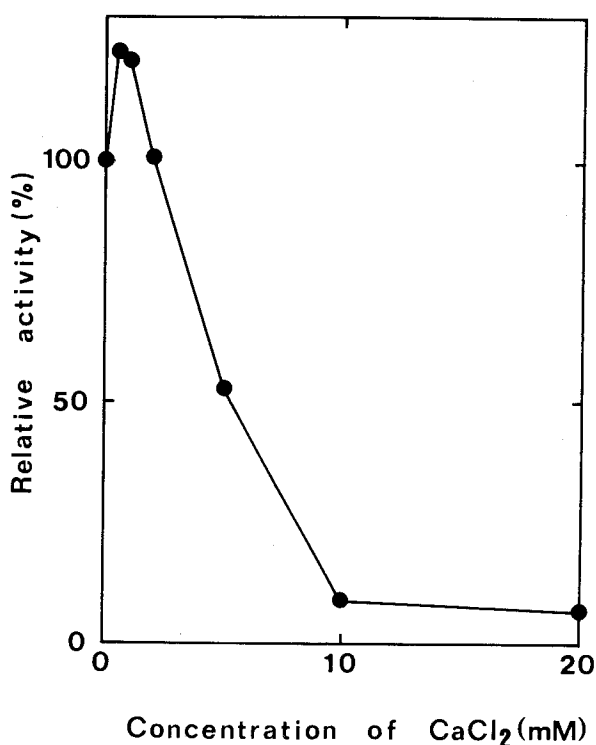


Fig. 29. Effect of  $\text{CaCl}_2$  on APendodeoxyribonuclease activity. The reaction mixtures contained 10 mM  $\text{MgCl}_2$ . The activity without  $\text{CaCl}_2$  was scored as 100 %.

When reaction mixture contained 10 mM  $\text{MgCl}_2$ , 5 mM EDTA did not inhibited the enzyme activity, whereas 20 mM EDTA inhibited completely the activity.

Among the other divalent cations tested,  $\text{Ca}^{2+}$  was not effective and therefore was unable to replace  $\text{Mg}^{2+}$ , although  $\text{Mn}^{2+}$  had a only slight activity (Table 5). When reaction mixture contained 10 mM  $\text{MgCl}_2$ , 10 mM  $\text{CaCl}_2$  completely inhibited the enzyme activity (Fig. 29).

Effect of ionic strength The enzyme activity is unusually resistant to the presence of NaCl in reaction mixtures. About 60 % of maximum

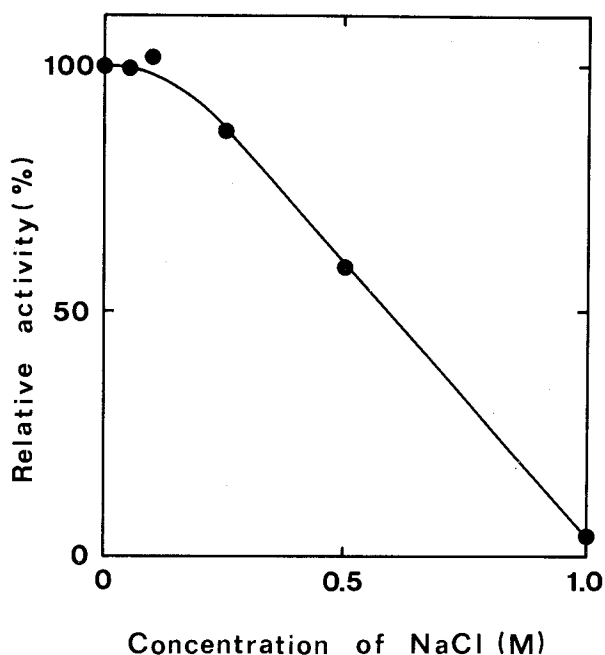


Fig. 30. Relative activity of APendodeoxyribonuclease (1.5 units) at different NaCl concentrations.

activity was retained in the presence of 0.5 M NaCl and the activity was completely inhibited by 1 M NaCl (Fig. 30).

Sulphydryl requirements The effect of sulphydryl reagents were summerized in Table 6. 2-mercaptoethanol was not effective to the enzyme activity. However, about 30 - 40 % of stimulation of the activity was observed in the presence of glutathione or dithiothreitol. In the absence of 2-mercaptoethanol in the reaction mixture, the enzyme activity was completely inhibited by addition of 1 mM p-chloromercuribenzoate but was not influenced by addition of 5 mM N-ethylmaleimide.

Effect of nucleotides Table 7 shows that APendodeoxyribonuclease

Table 6. Effect of sulphydryl reagents on the activity of APendonuclease.

Additions <sup>a</sup>	Concentration (mM)	Relative activity (%)
None	0	100
2-Mercaptoethanol	1	98
	2	102
	5	93
	10	86
Glutathione	0.1	103
	0.2	106
	0.5	129
	1	143
	2	102
Dithiothreitol	0.1	101
	0.2	119
	0.5	137
	1	122
	2	122
N-Ethylmaleimide	0.2	90
	0.5	88
	1	96
	2	87
	5	98
p-Chloromercuribenzoate-2Na	0.1	23
	0.2	19
	0.5	13
	1	7.5
	2	4.6

a, Activity was measured in the standard assay conditions, except that 2-mercaptoethanol was omitted from the assay mixture.

Table 7. Effect of different nucleotides on the activity of APendonuclease.

Additions <sup>a</sup>	Concentration (mM)	Relative activity (%)
None	0	100
ATP	0.01	107
	0.02	108
	0.05	113
	0.1	122
	0.2	120
dATP	0.5	105
	0.01	122
	0.02	134
	0.05	120
	0.1	127
	0.2	130
	0.5	130

a, Nucleotides were added to the standard assay mixture.

activity was increased slightly (10 to 30 %) in the presence of 0.01 to 0.5 mM of ATP or dATP.

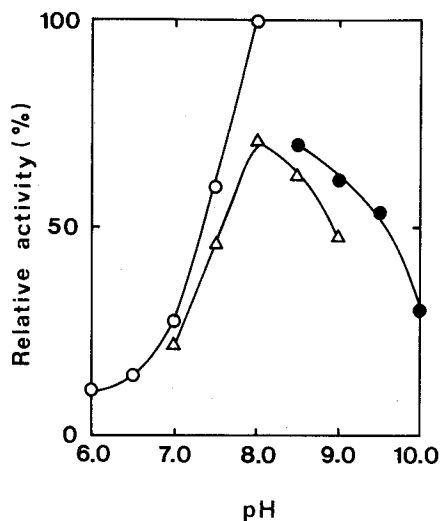


Fig. 31. Effect of pH on APendodeoxyribonuclease activity.

Activity was measured in the standard assay conditions, except that buffer A was replaced by 50 mM Tris-malate-KOH (O), 50 mM Tris-HCl (Δ), or 50 mM glycine-KOH (●). The activity in Tris-malate-KOH at pH 8.0 was set at 100 %.

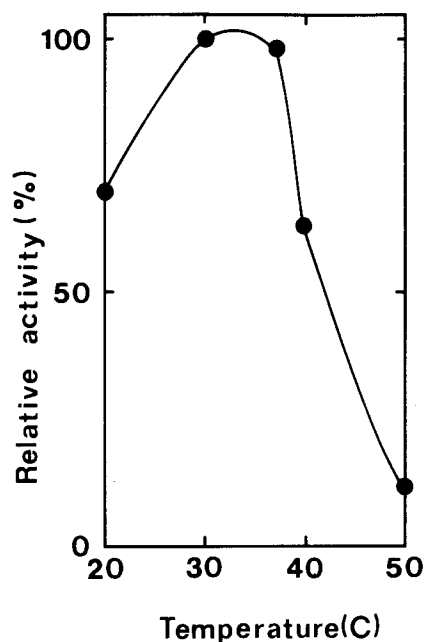


Fig. 32. Effect of reaction temperature on APendodeoxyribonuclease activity. Activity was measured in the standard assay conditions, except that reaction temperature was varied.

Effect of pH The optimum pH for the APendodeoxyribonuclease activity was examined using different buffer solutions ranging from pH 6.0 to 10.0 (fig. 31). The optimum pH for the activity of purified enzyme was 8.0.

Effect of temperature The effect of incubation temperature on the enzyme activity is shown in Fig. 32. The optimum temperature for the enzyme activity appears to be about 34°C.

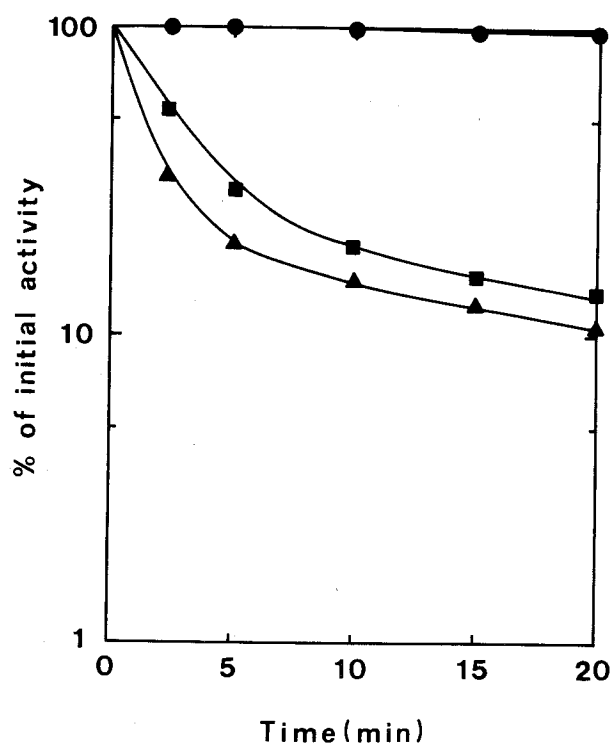


Fig. 33. Heat inactivation of APendodeoxyribonuclease.

APendodeoxyribonuclease was incubated at various temperatures for indicated time and immediately after the incubation, the enzyme activity was assayed. Symbols : ● , incubated at 0°C ; ■ , incubated at 37°C ; ▲ , incubated at 40°C.

#### Thermosensitivity of the enzyme

Fig. 33 shows the thermosensitivity of the enzyme activity at 0, 37, and 40°C. Aliquots (200  $\mu$ l) of Preparation VI (3 units) were heated at 37, or 40°C for 0 - 20 min in test tubes. Immediately after the heating, the remaining APendodeoxyribonuclease activity was assayed. The results, expressed as percentage of the initial activity, were plotted on a semilogarithmic scale. At a low enzyme concentration the purified enzyme (Prepara-



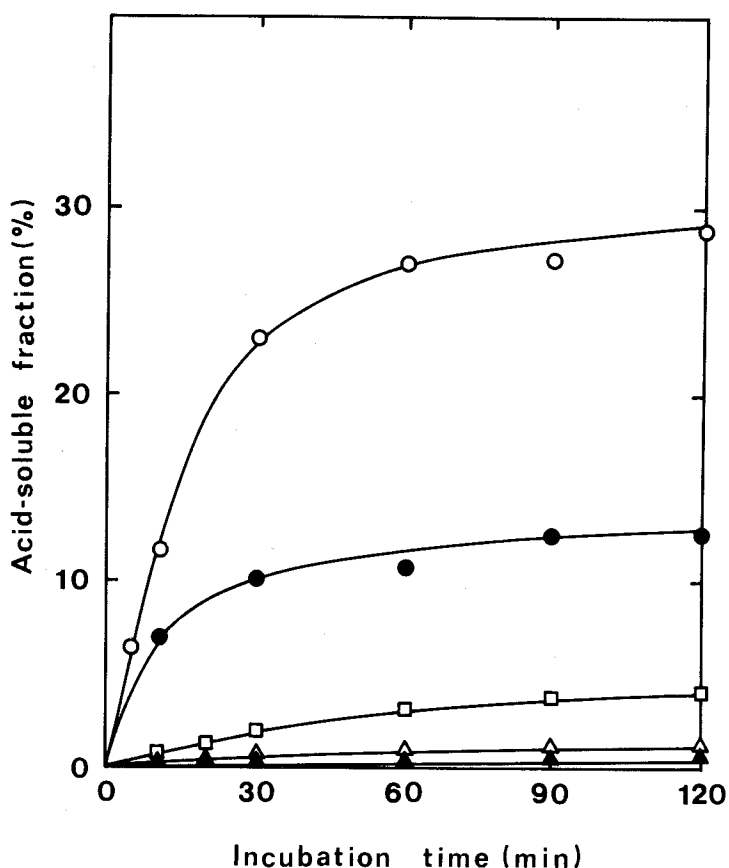


Fig. 34. Activities of the purified enzyme (Preparation VI) on native, denatured, alkylated, depurinated and single strand depurinated DNA. ( $\Delta$ ), native DNA ; ( $\blacktriangle$ ) denatured DNA ; ( $\square$ ), alkylated DNA ; ( $\bullet$ ), single strand depurinated DNA ; ( $\circ$ ), depurinated DNA. (Concentration of substrate DNA was 6.7  $\mu\text{g/ml}$ ). Aliquots were taken after 0 to 120 min incubation to measure the acid-soluble radioactivity which was plotted as fraction of the total radioactivity.

tion VI) was sensitive to heat ; the half lives of the enzyme at 37°C and 40°C were about 3 min and 1 min, respectively. However, the enzyme was stable at 0°C. The concentrated Preparation VI (DNA-

cellulose fraction) was stable for three months at -20°C in buffer A. However, the enzyme was easily inactivated by the repeated freezing and melting.

Substrate specificity      Preparation VI was mixed with  $^3\text{H}$ -labeled native DNA, single strand untreated DNA, alkylated DNA, single strand alkylated-depurinated DNA, and double strand alkylated-depurinated DNA, respectively, and incubated at 37°C ; aliquots were taken after 0 to 120 min incubation to measure the acid-soluble radioactivity. Figure 34 shows that Preparation VI has a remarkable activity to alkylated-depurinated DNA, but its activity to native, single strand, and alkylated DNA was very little. It is reported that endonuclease IV, VI of *E. coli* (52,53) and APendonuclease of *B. subtilis* (57) are specific to apurinic sites in double strand DNA and not active to single strand depurinated DNA. However, Preparation VI is also active to single strand alkylated-depurinated DNA. Its activity is about a half of that to double strand alkylated-depurinated DNA.

*E. coli* APendonuclease purified by Verly and his colleagues (endonuclease VI) (52,131) and *H. influenzae* APendonuclease purified by Clements et al. (54) have an exonuclease activity. Figure 34 shows that preparation VI's activity to native and single strand DNA is very low. In order to investigate details of the exonuclease activity, the exonuclease activity to  $^3\text{H}$ -labeled sonicated DNA of *B. subtilis* which was prepared with the method of Gossard and Verly(131) was measured in the presence or absence of  $\text{MgCl}_2$ . Consequently,

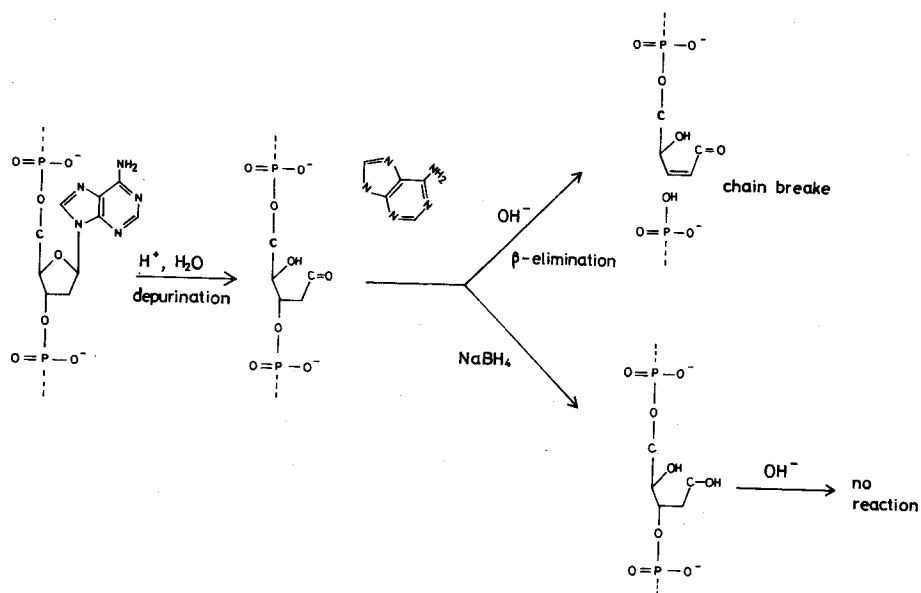


Fig. 35. Molecular mechanism of depurination and subsequent chain breakage.

Preparation VI has no exonuclease activity.

Enzymatic degradation of heated-reduced DNA It is reported that the deoxyribose residue at an apurinic or apyrimidinic sites in DNA exists in equilibrium between the furanose form and the aldehyde form. In alkaline solution, a  $\beta$ -elimination reaction consequently occurs. In this reaction phosphate is eliminated from the 3' position of an adjacent deoxyribose residue in its aldehyde form, so a chain break taken place at the 3' side of the lesion (118,137,138). Hadi and Goldthwait (130) reported that it was possible to stabilize the alkali-labile apurinic sites by adding reducing agent  $\text{NaBH}_4$ . Figure 35 shows molecular mechanism of apurinic sites,  $\beta$ -elimination, and reduction with  $\text{NaBH}_4$ .

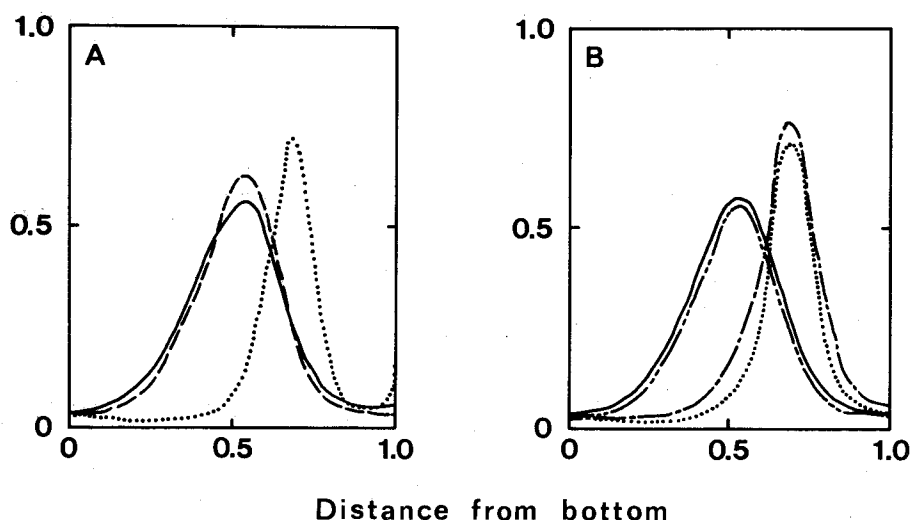


Fig. 36. Effect of NaOH and APendodeoxyribonuclease on heated-reduced DNA. *B. subtilis* DNA in SSC(pH 3.5) was heated at 60°C for 30 min and was cooled rapidly. After addition of potassium phosphate buffer (pH 6.5), NaBH<sub>4</sub> was added to reduce heated DNA. The solutions were kept at room temperature for 1 h and were dialyzed against SSC containing 10 mM MgCl<sub>2</sub>(pH 7.0) at 4°C for 12 h. Native or heated-reduced DNA was incubated for 30 min at 37°C with Preparation VI. Prior to analysis in alkaline sucrose gradients, all DNA solutions were mixed with 1/20 volume of 2 N NaOH and incubated at 37°C for 15 min. Each reaction mixture was layered on 5-20 % alkaline sucrose density gradients and centrifuged in an SW50.1 rotor at 40,000 rpm for 4.5 h at 5°C. (A) Alkaline sucrose gradient centrifugation of the native DNA (---), heated-reduced DNA (—), and heated (.....). (B) Alkaline sucrose gradient centrifugation of the native DNA + enzyme (— · —), heated-reduced DNA + enzyme (— · —), heated-reduced DNA (—), and heated DNA (.....).

In order to know whether this enzyme is able to act on very few apurinic sites in large DNA molecules, depurinated (heated at 60°C for 30 min at pH 3.5) and reduced DNA was mixed with the purified

enzyme and incubated at 37°C for 30 min. After incubation, the reaction mixture was treated with alkali and subjected to alkaline sucrose density gradient centrifugation. Native DNA and heated-reduced DNA showed the same sedimentation patterns (Fig. 36 A). About the same number of apurinic sites in the heated-reduced DNA were cleaved by the enzyme as were cleaved by alkaline treatment (Fig. 36 B). Single strand DNA breaks were not introduced in native DNA with alkali nor with the purified enzyme. These data show that this endonuclease specifically cleaves the heat-induced alkali-labile sites (i.e. apurinic sites) in DNA.

Table. 8. Effect of NaOH and APendonuclease on alkylated-depurinated reduced DNA.

Alkylated-depurinated [ $^3\text{H}$ ]DNA or alkylated-depurinated [ $^3\text{H}$ ]DNA reduced with  $\text{NaBH}_4$  was incubated for 15 min at 37°C with APendonuclease or NaOH. The acid-soluble radioactivity was measured and the results are expressed as fractions of the substrate radioactivity.

Substrate	Proportion of radioactivity in acid-soluble fraction	
	NaOH	APendo-nuclease
	(%)	(%)
Alkylated-depurinated DNA	39	24
Alkylated-depurinated reduced DNA	0.3	20

Determination of cleavage sites of APendodeoxyribonuclease      The following two methods were used for determination of the terminus at cleaved site of purified APendodeoxyribonuclease.

The first was according to the method of Gossard and Verly (131). One of two tubes containing 200  $\mu$ l alkylated-depurinated [ $^3$ H]DNA in SSC containing 0.1 M  $\text{MgCl}_2$  was reduced with sodium borohydride by the same method that was used for the reduction with sodium boro- $[\text{}^3\text{H}]$ -hydride of non-labeled depurinated DNA. The other was treated identically without sodium borohydride. Both solutions were subsequently dialyzed against SSC containing 0.01 M  $\text{MgCl}_2$  at 4°C. Aliquots (70  $\mu$ l) of the alkylated-depurinated [ $^3$ H]DNA reduced or not were mixed with 30  $\mu$ l of either Preparation VI, or 0.4 M NaOH, or SSC containing 0.01 M  $\text{MgCl}_2$  (control). After 15 min at 37°C the mixtures were cooled in ice water and the acid-soluble radioactivities were measured : the results obtained for the enzyme and NaOH were corrected for the control. Table 8 shows that reduced-depurinated DNA and depurinated DNA were similarly cleaved by APendodeoxyribonuclease ; on the other hand, reduction of depurinated DNA suppressed the action of NaOH.

In order to determine the cleavage site, apurinic sites in DNA were labeled with sodium boro- $[\text{}^3\text{H}]$ -hydride. After incubation with APendodeoxyribonuclease the apurinic DNA was denatured by heating at 100°C for 10 min. The cleaved apurinic DNA was incubated with snake venom or spleen phosphodiesterase after pretreatment with *E. coli* alkaline phosphatase (ALP). After 30 min incubation, cold perchloric acid was added to stop the reaction. The radioactivity in acid-

soluble fraction was determined.

Table 9 shows that snake venom phosphodiesterase, which hydrolyzes DNA strand from 3'-site to 5'-site, releases  $^3\text{H}$ -labeled apurinic sugar moiety of DNA regardless of the reaction mixtures are pretreated with ALP or not. On the other hand, spleen phosphodiesterase, which hydrolyzes DNA strand from 5'-site to 3'-site, releases little or no  $^3\text{H}$ -labeled apurinic sugar moiety whether DNA is pretreated with ALP or not. These results show that this APendodeoxyribonuclease of *B. subtilis* cleaves the 3'-side of apurinic sites in DNA strand

Table 9. Action of snake venom and spleen phosphodiesterase on apurinic DNA reduced with sodium boro- $^3\text{H}$ -hydride and treated with APendonuclease. Alkylated-depurinated DNA reduced with sodium boro- $^3\text{H}$ -hydride was incubated with APendonuclease at 37°C for 30 min and subsequently denatured by heating at 100°C for 10 min. Denatured DNA was incubated with snake venom phosphodiesterase (0.4 unit) or with spleen phosphodiesterase (2 units), after a treatment with alkaline phosphatase (2 units). The acid-soluble  $^3\text{H}$  was determined and the results were expressed as percentage of total radioactivity in the substrate DNA ; correction is made for a control.

ALP	Phosphodiesterase	Acid-soluble fraction ( % )	Corrected ( % )
-	None	30	0
-	Snake venom	49	19
+	Snake venom	52	22
-	Spleen	29	0
+	Spleen	36	6

giving a 3'-OH and 5'-phosphate.

To confirm this result, another method was used. The DNA containing apurinic sites was incubated with APendodeoxyribonuclease at 37°C for 30 min or heated in the Hepes-KOH buffer (pH 7.9) at 70°C for 10 h to break the apurinic sites in DNA. After breakage of the apurinic sites, the DNA was denatured by formamide. After being treated with ALP. DNA was incubated with [ $\gamma$ - $^{32}\text{P}$ ]ATP (0.3 mCi/ $\mu\text{mol}$ ) and polynucleotide 5'-hydroxyl-kinase. The reaction was stopped with addition of cold trichloroacetic acid.  $^{32}\text{P}$  incorporated into acid-insoluble fraction was determined. Table 10 shows that only when the apurinic DNA which is cleaved with APendodeoxyribonuclease or heat is pretreated with ALP  $^{32}\text{P}$  was remarkably incorporated into acid-insoluble fraction. Lindahl and Anderson (118) reported that when the DNA containing apurinic sites was heated in neutral pH solution, chain breaks were predominantly due to  $\beta$ -elimination and occurred at the 3'-side of apurinic sugar moiety giving a 3'-OH and 5'-phosphate. Therefore, if the 5'-side of apurinic sugar residue was phosphorylated with [ $\gamma$ - $^{32}\text{P}$ ]ATP and polynucleotide 5'-hydroxyl-kinase the apurinic sugar residue would be split off by  $\beta$ -elimination at 100°C for 60 min in neutral pH solution and  $^{32}\text{P}$  would be released in acid-soluble fraction. In this experiment,  $^{32}\text{P}$  incorporated into DNA was not released in acid-soluble fraction even after incubation at 100°C for 60 min under neutral pH. Therefore, these data show that phosphorylated terminal residues were nucleotides but not



apurinic sugar moieties.

The results shown in Tables 9 and 10 indicate clearly that the APendodeoxyribonuclease of *B. subtilis* cleaves the DNA strands on the

Table 10. End group analysis after enzymatic cleavage at apurinic sites in DNA.

Calf thymus DNA (1 mg/ml) in SSC (0.15 M NaCl/0.015 M sodium citrate, pH 5.0) was incubated for 8 h at 70°C to obtain 1 % depurination of the DNA. To obtain chain breaks at the apurinic sites, depurinated DNA was treated with APendonuclease or heated at 70°C for 10 h in 0.09 M Hepes-KOH/0.02 M MgCl<sub>2</sub>/0.1 M glycine (pH 7.9). Control DNA was also incubated in the enzyme reaction mixture, except that no enzyme was added. All DNA solutions were dialyzed at 4°C against 95 % formamide and denatured at 37°C for 2 h and dialyzed at 4°C successively against 0.02 M NaCl/0.01 M Tris-HCl (pH 8.0)/6 % formaldehyde and then dialyzed against the same buffer without formaldehyde and each DNA concentration was determined. One-half of each solution was treated with *E. coli* alkaline phosphatase (ALP, 10 µg/ml) for 30 min at 37°C. To inactivate ALP, EDTA was added (5 mM) and the mixture was heated at 100°C for 2 min. All DNA solutions were then incubated with polynucleotide 5'-hydroxyl-kinase at 37°C for 45 min, in the following reaction mixture (100 µl) : 0.07 M Tris-HCl (pH 7.5), 0.01 M MgCl<sub>2</sub>, 0.01 M 2-mercaptoethanol, 0.02 M K<sub>2</sub>HPO<sub>4</sub>, 2 x 10<sup>-5</sup> M [γ-<sup>32</sup>P] ATP (0.3 mCi/µmole), calf thymus DNA (35 µg/ml), and polynucleotide 5'-hydroxyl-kinase (5 units). After the reaction was stopped by chilling, one set of sample was added 100 µl of calf thymus DNA (2 mg/ml) and precipitated with 0.5 ml of 7.2 % trichloroacetic acid. The precipitates were collected on Millipore filter (type GS 0.22 µm), washed with cold 7.2 % trichloroacetic acid, dried, and analyzed for radioactivity. While duplicate samples were heated at 100°C for 60 min before cold trichloroacetic acid precipitation.

Apurinic DNA	<sup>32</sup> P incorporated at cleaved apurinic sites		
	without ALP	with ALP	with ALP and heated at 100°C for 60 min
		p moles	
Unhydrolyzed	4.4	10.3	8.6
Hydrolyzed with heat (70°C, 10 h)	7.8	34.0	23.0
Hydrolyzed with APendonuclease	8.0	45.9	42.4

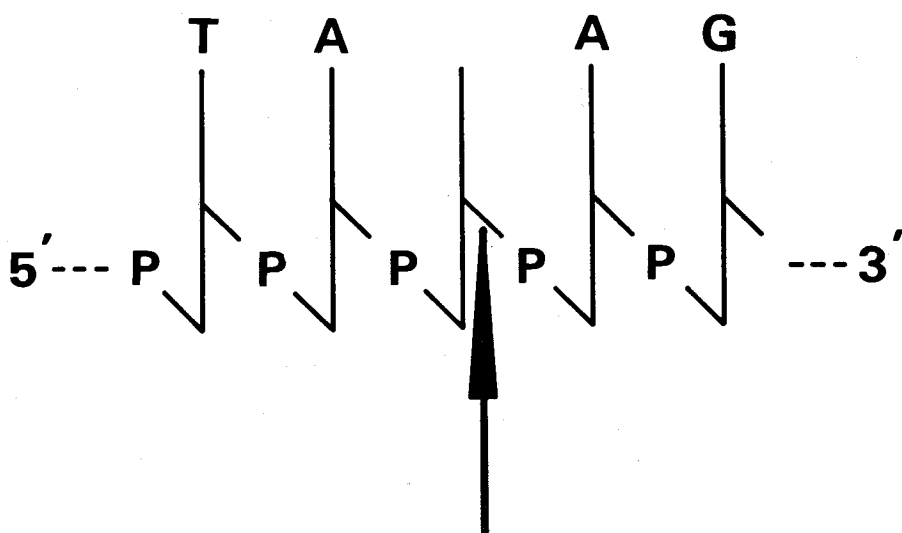


Fig. 37. Cleavage site of APendodeoxyribonuclease.

3'-side of the apurinic sugar residue giving a 3'-OH and a 5'-phosphate (Fig. 37).

Gossard and Verly (113) reported that APendodeoxyribonuclease of *E. coli* (endonuclease VI) cleaves the DNA strand on the 5'-side of the apurinic sites giving a 3'-OH and 5'-phosphate. On the other hand, Ljungquist and Lindahl (61) demonstrated that APendonuclease from calf thymus incises the DNA strand on the 3'-side of apurinic sites. This result suggests that the incision mechanism of APendodeoxyribonuclease from *B. subtilis* is similar to that of calf thymus APendodeoxyribonuclease.

#### APendodeoxyribonuclease level during the life cycle of *B. subtilis*

The levels of APendodeoxyribonuclease activity in the intact spores, germinating spores, vegetative cells, and sporulating cells of *B.*

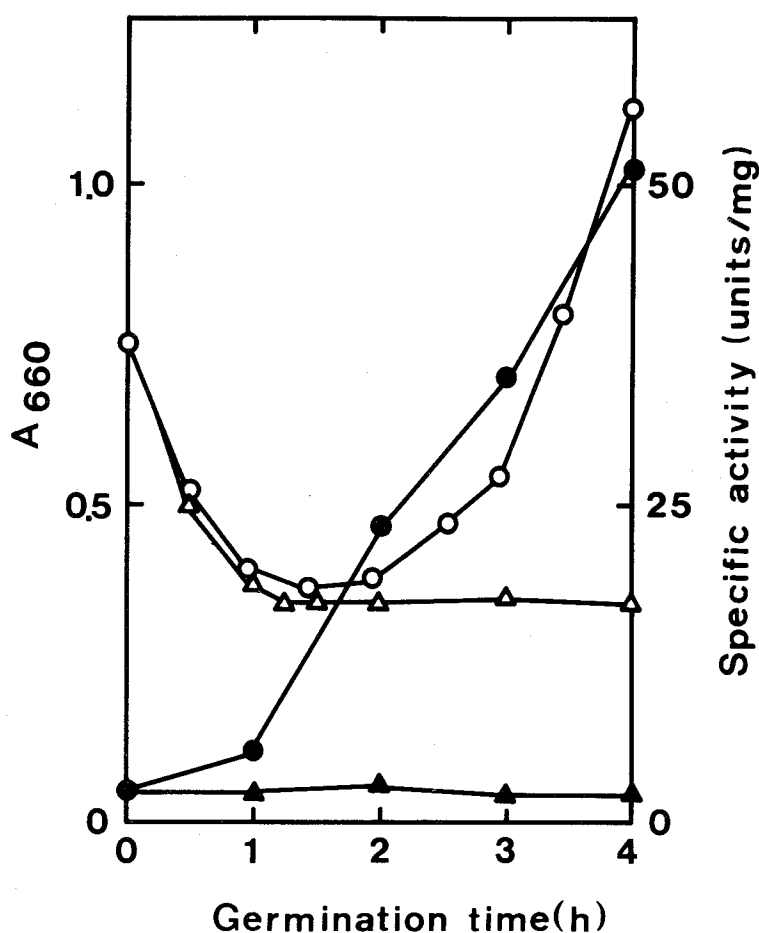


Fig. 38. Changes in APendodeoxyribonuclease activity during germination of *B. subtilis* spores. Symbols : ○ , absorbance at 660 nm (control) ; △ , absorbance at 660 nm of germinating spores containing chloramphenicol (50  $\mu$ g/ml) ; ● , APendodeoxyribonuclease activity (control) ; ▲ , APendodeoxyribonuclease activity of germinating spores containing chloramphenicol (50  $\mu$ g/ml).

*subtilis* 168Tt were measured. Cultures were grown at 37°C in the Schaeffer liquid nutrient broth sporulation medium. Cells were harvested at approximately one doubling time before and at 0, 2, 4, and

Table 11. Variation of APendodeoxyribonuclease level during germination, vegetative growth and sporulation of *B. subtilis*.

Growth stage		Total activity (units)	Total protein (mg)	Specific activity (units/mg)
<b>Spore</b>				
Germination	0 h	35	10.5	3.3
	1	76	11.6	6.6
	2	315	13.6	23.2
	3	993	28.4	35.0
	4	3101	60.0	51.7
<b>Vegetative cell</b>				
Log phase	OD = 0.5	9603	78.4	116
stationary phase	t <sub>0</sub>	4188	42.0	100
	t <sub>2</sub>	5438	52.4	104
	t <sub>4</sub>	5625	48.8	115
	t <sub>8</sub>	3125	44.0	71

8 h after the end of exponential growth. Spores were prepared as described in Chapter III. Spore germination was carried out in the Schaeffer's medium. Germinating cells were harvested at the intervals of every 1 h after the start of germination. The cells were disintegrated by sonication with glass beads for 15 min using the method described by Cobianchi et al. (139). The supernatant fluid obtained by the second centrifugation was used for the enzyme assay.

As shown in Fig. 38, spores have little APendodeoxyribonuclease activity. The activity appears in germinating spores being incubated for 1 h in the Schaeffer's medium. After that, the activity increases rapidly. This process is corresponding to the DNA synthesis during germination (Chapter IV). However, as the APendodeoxyribo-

nuclease activity does not appear during germination in the presence of chloramphenicol which is an inhibitor of protein synthesis (Fig. 38), APendodeoxyribonuclease must be synthesized *de novo* during germination.

The maximum APendodeoxyribonuclease level is observed in the middle log phase cells. The enzyme activity does not change during sporulation until  $t_4$  stage. After  $t_4$  stage the activity in the cells extracts decreases. These results are summarized in Table 11.

#### 4. DISCUSSION

In the previous chapter, it was found that acid-heat treatment caused the single strand scissions of DNA in spores. Considering from the experimental results on the depurination of purified DNA the depurination is responsible for *in vivo* DNA scissions. Then, in case that most of the heated spores are survival the apurinic sites of DNA in spores caused by acid-heating are restored during germination.

Therefore, in order to understand the mechanisms of DNA damage (depurination) by heating and its repair system, the enzyme activity specific to apurinic DNA induced by acid-heating was investigated.

Consequently, an endodeoxyribonuclease which hydrolyzes DNA containing apurinic sites was purified from *B. subtilis* vegetative cells at approximately 8,800-fold with a yield of 22 %. The final prepa-

ration (Preparation VI ; DNA-cellulose fraction) gave a single protein band corresponding to the position of the enzyme activity on the gel electrophoresis. By use of Sephadex G-200 and glycerol gradient centrifugation, it was found that APendodeoxyribonuclease has molecular weight of 105,000 and that this enzyme was dissociated to multiple identical subunits having molecular weight of 26,000 on SDS electrophoresis. Those results suggest that APendodeoxyribonuclease is composed of four identical subunits.

Comparing with APendodeoxyribonuclease from *E. coli* (IV, M.W. 33,000 ; VI, M.W. 32,000), *B. stearothermophilus* (M.W. 28,000) and *B. subtilis* (M.W. 56,000) molecular weight of this enzyme is much higher than those of others (52,53,57,58).

The activity of the purified enzyme is strictly restricted to apurinic sites in double or single strand DNA and not active on native, denatured, or alkylated DNA. Previously reported APendodeoxyribonucleases were not active to apurinic sites in single strand DNA.

The purified enzyme strictly requires  $Mg^{2+}$  for its activity.  $Mn^{2+}$  and  $Ca^{2+}$  were not effective. However, the enzyme reported by Inoue and Kada requires no divalent cations and is insensitive to EDTA (57). Resistance of the purified enzyme to NaCl is higher than that of APendodeoxyribonuclease reported by Inoue and Kada (57).

Considering its strict requirement of  $Mg^{2+}$ , inhibition by EDTA, resistance to NaCl, substrate specificity, and the molecular weight,

it is thought that the endonuclease specific to apurinic acid DNA isolated from *B. subtilis* is entirely different from that of *B. subtilis* reported by Inoue and Kada (57) and also endonuclease IV and VI of *E. coli* (52,53).

By the end group analysis it was found that the purified APendodeoxyribonuclease of *B. subtilis* cleaves of DNA strands on the 3'-side of the apurinic sugar residue giving a 3'-OH and a 5'-phosphate. However, the endonucleolytic cleavage of endonuclease VI (=exonuclease III) of *E. coli* generates a 3'-OH nucleotide end and a base-free deoxyribose 5'-phosphate at the 5'-terminus (131). On the other hand, APendodeoxyribonuclease from calf thymus (61) incised the DNA strand on the 3'-side of apurinic sites. It is suggested that the incision mechanism of APendodeoxyribonuclease of *B. subtilis* is similar to that of calf thymus APendodeoxyribonuclease.

Initially the apurinic sites are cleaved by APendodeoxyribonuclease, and then the deoxyribose-5'-phosphate residue, and possibly a small number of additional residue, are released by exonuclease action, and the DNA is finally subjected to repair replication and ligation as in the original excision repair model. The individual steps of repair process in a *B. subtilis* system are schematically shown in Fig. 39. Since the side of cleavage of APendodeoxyribonuclease from *B. subtilis* is different from that of *E. coli* endonuclease VI, it is thought that the catalytic mechanism of exonucleases are different.

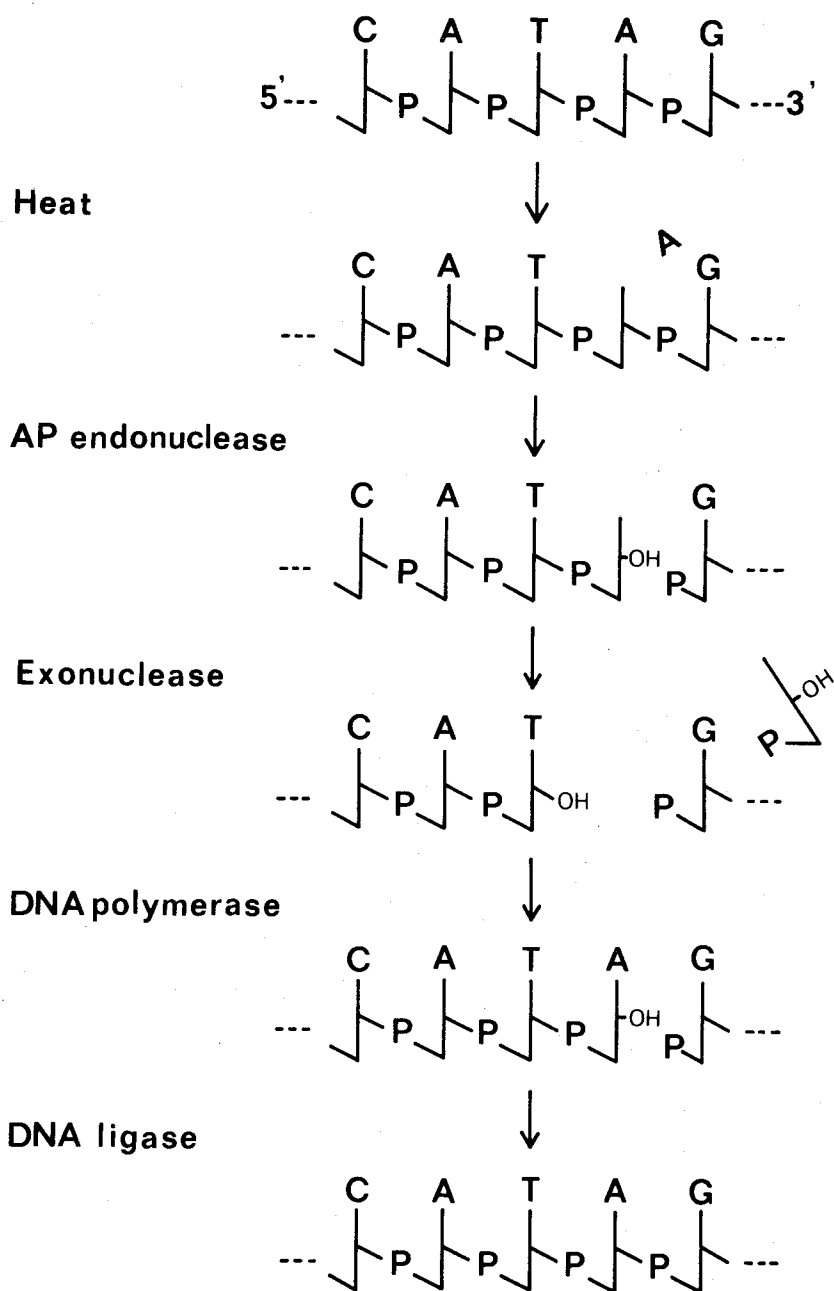


Fig. 39. Scheme for repair process of heat-induced depurinated DNA.



As shown in Chapter III and IV, acid-heat treatment of *B. subtilis* spores induced the depurination of DNA. The depurinated DNA of spores which was heated at 70°C for 20 min (pH 5.0) was repaired completely within 2 h after germination. As shown in Fig. 38, spores have very little APendodeoxyribonuclease activity and this activity increases rapidly after 1 h from germination. As DNA synthesis of the acid-heated spores begins after 90 min of incubation in germination medium, it is strongly suggested that APendodeoxyribonuclease synthesized during germination contributes to the repair mechanism of apurinic/apyrimidinic sites of DNA in spores.

## 5. SUMMARY

A new endodeoxyribonuclease specific to apurinic sites in DNA was purified from vegetative cells of *B. subtilis*. The enzyme hydrolyzes a phosphodiester bond at the heat-induced apurinic sites in double or single strand DNA ; it does not hydrolyze native nor alkylated DNA. The endonuclease has a molecular weight of around 105,000 and consists of four identical subunits. The enzyme absolutely requires  $Mg^{2+}$  for its activity and is inhibited by EDTA. It is completely inhibited by 1 M NaCl or 1 mM p-chloromercuribenzoate. The APendodeoxyribonuclease of *B. subtilis* cleaves the DNA strands on the 3'-side of the apurinic sugar residue giving a 3'-OH and 5'-phosphate. These properties are very different from those of the

endonuclease specific to apurinic sites in DNA isolated from *B. subtilis* by Inoue and Kada and those of endonuclease IV and VI of *E. coli*.

The maximum APendodeoxyribonuclease level is observed in the middle log phase cells. The enzyme activity does not change during sporulation until  $t_4$  stage and after  $t_4$  stage it decreases. Spores have little APendodeoxyribonuclease activity. But the activity increases rapidly after 1 h from germination. Since the repair of depurinated DNA in spores, DNA synthesis, and appearance of APendodeoxyribonuclease activity are found almost at the same time, it is strongly suggested that APendodeoxyribonuclease synthesized during germination contributes to the repair mechanism of apurinic sites of DNA in spores.

## Chapter VII

### SUMMARY AND CONCLUSIONS

In order to understand the mechanisms involved in the DNA injury by heat treatment and its repair, *in vivo* and *in vitro* DNA damages of *B. subtilis* heated under various conditions were studied. And the enzyme acting on heat-injured DNA was purified and characterized.

The results obtained are summarized as follows :

- (1) Spores of *B. subtilis* injured by the heat treatment as 90°C for 10 min could not form colonies on Demain's agar medium unless supplemented with glycine, threonine, or homoserine ; the heated *B. subtilis* became auxotrophs requiring these amino acids. These amino acids requirements were genetically inherited.

Therefore, it is suggested that the appearance of auxotrophic mutants of spores heated at 90°C for 10 min is due to the minor modification of DNA molecules by heat, such as depurination in spores.

- (2) The effect of pH on the thermal damages of spores of *B. subtilis* was observed. The results indicate that at the lower pH and the higher temperature, the more the spores were inactivated.

To know the effect of acid-heating on DNA in spores, DNA in the heated spores was extracted and analyzed by alkaline sucrose gradient sedimentation. At neutral pH, single strand scissions in

DNA did not occur after heating for 30 min at 60, 70, 80, and 90°C, but at low pH DNA breaks increased according to the increasing acidity. These results show that at the lower pH and the higher temperature the spores were heated, the more the single strand scissions of DNA in spores occurred.

The results of studies on the temperature and pH dependence of depurination of *in vitro* DNA indicated that depurination increased with decreasing pH values and increasing temperature. The acid-heat induced DNA injury of spores corresponded to that of depurination of DNA heated at low pH *in vitro*. Therefore, it is concluded that DNA in spores of *B. subtilis* was depurinated by acid-heat treatment, but at neutral pH depurination of DNA in spores was scarcely occurred by heating at 60 - 90°C. Sedimentation analysis indicated that in the vegetative cells heating at 50°C induced the single strand breakages of DNA molecules. However, DNA breakages in vegetative cells did not depend on the heating temperature or the heating time. Since the heat-induced DNA breakages were inhibited by p-CMB, it is concluded that DNA in vegetative cells was destroyed by endonuclease, which might be released or activated by mild heating.

- (3) When the spores of *B. subtilis* which were heated at 70°C for 20 min at pH 5.0, by the condition of which the survivals were not greatly reduced, were incubated in germination medium at 37°C for 120 min, the apurinic sites in DNA were restored. This DNA re-

pairing process corresponds to the DNA synthesis during germination.

- (4) As the result of investigation of the enzyme acting on heat-injured DNA, a new endodeoxyribonuclease specific to apurinic sites in DNA was purified from vegetative cells of *B. subtilis*. The enzyme hydrolyzes a phosphodiester bond at the heat-induced apurinic sites in double or single stranded DNA, but has no action on normal double stranded DNA, single stranded DNA, nor alkylated DNA. It is composed of four identical subunits and has a molecular weight of about 105,000 daltons. It strictly requires  $Mg^{2+}$  for its activity and is sensitive to EDTA. It is completely inhibited by 1 M NaCl or 1 mM p-chloromercuribenzoate. By the end group analysis, it was found that APendodeoxyribonuclease of *B. subtilis* cleaves the DNA strands on the 3'-side of the apurinic sugar residue giving a 3'-OH and 5'-phosphate. These properties are very different from those of the APendodeoxyribonuclease isolated from *B. subtilis* by Inoue and Kada and those of endonuclease IV and VI of *E. coli*.

APendodeoxyribonuclease level is at its maximum in the middle log phase cells. The enzyme activity does not change during sporulation until the  $t_4$  stage. After the  $t_4$  stage the activity in the cell extracts decreases. The APendodeoxyribonuclease activity is very little in spores and synthesized during germination. The enzyme activity appears after germination for 1 h and after that, the activity increases rapidly. This process corresponds to the

DNA synthesis during germination.

These results strongly suggest that apurinic sites in DNA of spores caused by acid heating are nicked by APendodeoxyribonuclease synthesized during germination and repaired by the subsequent excision repair system.

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